Early events during *Listeria monocytogenes* infection: ups and downs of chemokines

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1 Introduction

1.1 The immune system

The immune system of vertebrates is designed to protect the host from infections by pathogenic microorganisms and consist of two distinct recognition systems: innate and adaptive.

The innate immune response is made up of a network of cells and soluble factors that defend the host in the first days against invading pathogenic microorganisms. Soluble components (e.g. defensins, complement, lysozym or type I interferons) as well as cellular components (e.g. phagocytic cells and NK cells) act as a first line of defense against the microorganisms intruding the body. The cells of the immune system react against molecules, which are basically distinct for the pathogen, like the cell wall lipopolisaccharide (LPS) or lipopoliteichoic acid (LTA). Such pathogen recognition is often based on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)

The adaptive immunity is mediated by T and B lymphocytes bearing clonally distributed antigen receptors. The specificities of these receptors are generated by somatic mechanisms, and therefore are not products of natural selection directed by pathogens. The reactivity of T and B cells is absolutely dependent on signals that are provided by the innate recognition system demonstrating the close interdependence of both systems. Thus, in the early phase of infection the innate immune system is essential to keep the infection in check, but also to control the adaptive response. A good example of this is the differentiation the two classes of helper T cells (Th1 and Th2).

1.1.1 The cellular components of the innate immune system

Like all other hematopoietic cells, the cells of the innate immune system, originate from stem cells in the bone marrow. Some develop into myeloid cells, a group typified by the large, cell- and particle- devouring white blood cells known as phagocytes. Phagocytes include monocytes and neutrophils (described in more detail below). Monocytes in blood migrate into tissue and differentiate into macrophages and dendritic cells. However, DCs can also be of lymphoid origin (Schotte et al. 2003). All those cells are also described below in more detail.

Additional myeloid descendants become granule-containing inflammatory cells such as eosinophils, basophils and mastcells. There are effector cells in parasitic infections and also play a crucial role in allergic responses.

1.1.1.1 Phagocytic cells

Phagocytes are a heterogeneous group of cells, including mononuclear phagocytes and neutrophils, functionally defined as cells that are able of microorganisms.

Phagocytosis

Phagocytosis is a process utilized by mononuclear phagocytes and neutrophils to ingest and clear large particles (>0.5μm). Particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. This leads to the polymerization of actin at the site of ingestion, and the internalization of the particle via an actin-based mechanism (Allen and Aderem 1996). Phagocytosis of microorganisms is a key element in host defense against bacterial infections. There are two kinds of phagocytosis:

- opsonin-dependent: immunoglobulins or complement factors act as an opsonin, thereby promoting ingestion via Fcγ or complement receptors (Wright SD and Silverstein 1986)
- opsonin-independent: ligands on the surface of microorganisms are directly recognized by receptors on the plasma membranes of phagocytes (Ofek et al. 1995).

The mechanisms that act during phagocytosis can also be divided into two types (Swanson and Baer 1995):

- zipper-like mechanism – phagocytosis mediated by macrophage Fc or complement receptors. Bacteria gradually sink into diplike structures of the host cell surface. During this process the target cell membrane closely surrounds the bacterial cell and does not form membrane ruffles. This type of phagocytosis is characteristic for the uptake of *L. monocytogenes* (Kuhn and Goebel 2000).
- macropinocytosis (trigger mechanism) – internalization of molecules into large spacious phagosomes morphologically identical to microphagosomes (Alpuche-Aranda et al. 1994). The ruffling of the host cell membrane associates this process and the formed

phagosomes are not closely associated with the ingested particle. This type of phagocytosis is characteristic for the *S. typhimurium* uptake (Amyere et al. 2002).

To exert their function, phagocytic cells have receptors able to recognize surface components of the pathogens (e.g. scavenger receptors, TLRs), on the other hand they also carry receptors for ingesting opsonized particles (e.g. integrins: CD11b/CD18, CD11c/CD18). Many of these receptors not only induce phagocytosis but also activate the cell to secrete cytokines, important regulatory compounds of the innate response (see below). In addition, the receptors also induce the release of effector molecules like toxic oxygen radicals, peroxides, nitric oxide and lipid mediators of inflammation such as prostaglandins and leukotrienes (Ezekowitz et al. 1991;Bevilacqua 1993;Fraser et al. 1998). After uptake and processing of the microbial antigen, macrophages and DCs can activate antigen specific T cells as well as B cells.

Macrophages

The phagocytes of the innate immunity system, described for the first time in 1884 by Metschnikoff provide the first line of defense against microorganisms. Monoblasts are the most immature cells committed to the monocyte lineage (Valledor et al. 1998). They divide and give rise to promonocytes and monocytes, which then enter the circulation and migrate to tissues. In response to particular stimuli monocytes can differentiate into macrophages (Valledor et al. 1998)

Macrophages express a variety of functions including phagocytosis, processing and presentation of antigens to T lymphocytes, production of cytokines, killing of microbes and tumor cells (Rutherford et al. 1993). Macrophages at different body sites are often represented by cells in different stages of maturation and activation, which results in morphological, functional and metabolic diversity (Rutherford and Schook 1992).

This macrophage heterogeneity is the result of a large number of factors, including the developmental stages that macrophages pass through from the resting state to an activated state (Rutherford et al. 1993). The genetic background of a mouse strain as well as availability of cytokines might contribute to the activation status of macrophages (Pace et al. 1985a;Pace et al. 1985b;Hogan and Vogel 1987). Recent evidence suggest that heterogeneity may result also from diversity among the progenitors (Leenen and Campbell 1993) Differences in phagocytic abilities as well as bactericidal activities among various macrophage subpopulations depend on differential expression of Fc and other surface receptors. To date, understanding of the origin and mechanisms of this macrophage heterogeneity is hampered by

the lack of appropriate cell surface markers that are characteristic for the different maturation and activation stages.

Neutrophils

Neutrophils (neutrophilic granulocytes) are the most numerous and the most important cellular component of the innate immune response. Mature neutrophils have an extremely short half live of ½ day. Thus, most of the cells produced by the bone marrow are neutrophils (Rosales and Brown EJ 1993). Similarly to macrophages, neutrophils can recognize and engulf many pathogens, even those not previously opsonized by antibodies or complement. Usually neutrophils represent the earliest phagocytes migrating into the sites of inflammation. In listeriosis for instance, the infective foci during the first two days of infection are rich in neutrophils. The usual time of neutrophil appearance in the infected organs is 24-48 h (Unanue 1997). Once there, they control local infection and the spread of the infection to the other sites (Rogers et al. 1994). In addition to phagocytosis, neutrophils produce bacteriostatic and toxic product and are able to eliminate a broad spectrum of pathogens.

Dendritic cells

DCs are specialized, migratory phagocytes that are found in blood, peripheral tissues and lymphoid organs. Immature DCs posses the unique ability to recognize and phagocytose antigens, and subsequently migrate to secondary lymphoid organs to induce resting T and B cells to respond (Banchereau et al. 2000;Jung et al. 2002). During migration the DCs mature and are then able to efficiently stimulate T cells. (Banchereau and Steinman 1998). Thus, they are cells playing a pivotal role in connecting innate and adoptive immunity and initiating adoptive immune responses (Taki 2002).

Dendritic cell subsets can be distinguished by their morphology, characteristic anatomic localization, expression of cell surface markers, produced cytokines and their ability to respond to different activating stimuli. Every subset probably has different function in the immune system. To date 6 major DC subsets have been described in mice (Ardavin 2003):

- $CD8^-$ - classic myeloid tissue DCs (Nakano et al. 2001)
- $CD8^+$ - lymphoid related DCs (Liu et al. 2002)
- $CD8^{int}$ - subclass of lymphoid DC's

- Langerhans cells (LCs) – appear to be a variant of $CD8^-$ cells specialized for the epidermis
- Dermal DCs (Ardavin 2003)
- $B220^+$ DCs - a recently identified member of the DC lineage, similar to thymic $CD8^+$ cells and therefore proposed to be of lymphoid origin (Vremec et al. 1992). They are not found in the peripheral tissues, but localized within T cell zones of lymphoid organs (Steinman et al. 1997). These cells are known to express type I interferons after stimulation (Asselin-Paturel et al. 2001). Moreover, plasmacytoid DCs (pDCs) express a distinct pattern of Toll-like receptors suggesting that they might have developed through different evolutionary pathways to recognize different microbial antigens. (Schotte et al. 2003). Murine pDCs are $CD11c^+$ $B220^+Gr1^+$, large, round cells with diffuse nuclei and rare dendrites.

Human monocyte-derived DCs have been shown to be able to phagocytose *L. monocytogenes*, probably in opsonin-dependent manner (Paschen et al. 2000). *Listeria* infected human DCs are able to survive infection for a considerable time (Kolb-Maurer et al. 2000). In contrast, mouse DCs have been found to undergo apoptosis after *L. monocytogenes* infection (Guzman et al. 1996). Maturation of DCs decreases their ability of bacterial uptake (Paschen et al. 2000).

1.1.1.2 NK cells

Natural killer cells are of lymphoid origin. Although closely related to T cells, these cells do not exhibit antigen specific receptors and are regarded as part of the innate immune system. They exhibit the innate ability to recognize infected cells, possibly by reacting against stressed cells (Orange et al. 1995). Without prior sensitization they can rapidly reject bone marrow transplants and tumor cells (Trinchieri 1989). NK cells have two roles during the early stage of infection: i) they kill infected cells directly; ii) they produce cytokines such as IFNγ, which then influences the immune response through activation of other cell-types.

NK cell migration towards infections *in vivo* can be induced by several chemokines like CCL3 or CXCL10 (Allavena et al. 1996;Salazar-Mather et al. 2002) (Taub et al. 1995), (Smyth et al. 1998). Type I IFNs, TNFα or IL-12 have been shown to activate NK cells (Pilaro et al. 1994;Orange et al. 1995;Biron 1997) to become cytotoxic and produce IFNγ (Natuk and Welsh 1987) (Bancroft 1993). In turn, IFNγ produced by stimulated NK cells can activate macrophages during the early phases of *L. monocytogenes* (Andersson et al. 1998). It

may also be important in the later $CD4^+$ T cell differentiation towards the Th1 subset (Scharton and Scott 1993)

1.1.2 The anatomy of the secondary lymphoid organs

The initiation of immune reactions requires the interaction of several cells and cell types. To allow such interreactions, they take place in the secondary lymphoid organs, where interactions are facilitated due to highly organized structures. These secondary or peripheral lymphoid organs are distinct from the primary or central lymphatic organs – the thymus and bone marrow – where the ontogeny of T cells and B cells takes place. Secondary organs are spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT). In this case only the spleen is relevant since it, besides the liver, is the primary target for *L. monocytogenes*.

Spleen

In most species the spleen is the largest collection of lymphoid cells in the body. This organ is designed to facilitate immune responses to antigens that have gained access to blood.

Spleen has several essential functions:

- similar to other secondary lymphoid organs, the spleen is the site of formation of activated, antigen specific lymphocytes, which then pass into the blood.
- the destruction place of damaged or aged red blood cells
- the storage of blood (and in blood cell formation during early fetal development).

Blood flow through the spleen is not organized in closed circulation. Rather the spleen represents an open sinus. Thus, circulating blood and microorganisms, or other molecules within blood, come into close contact with macrophages and lymphocytes, which are abundant in this organ. The anatomy of the spleen therefore ensures the contact of antigen with antigen specific defense mechanisms.

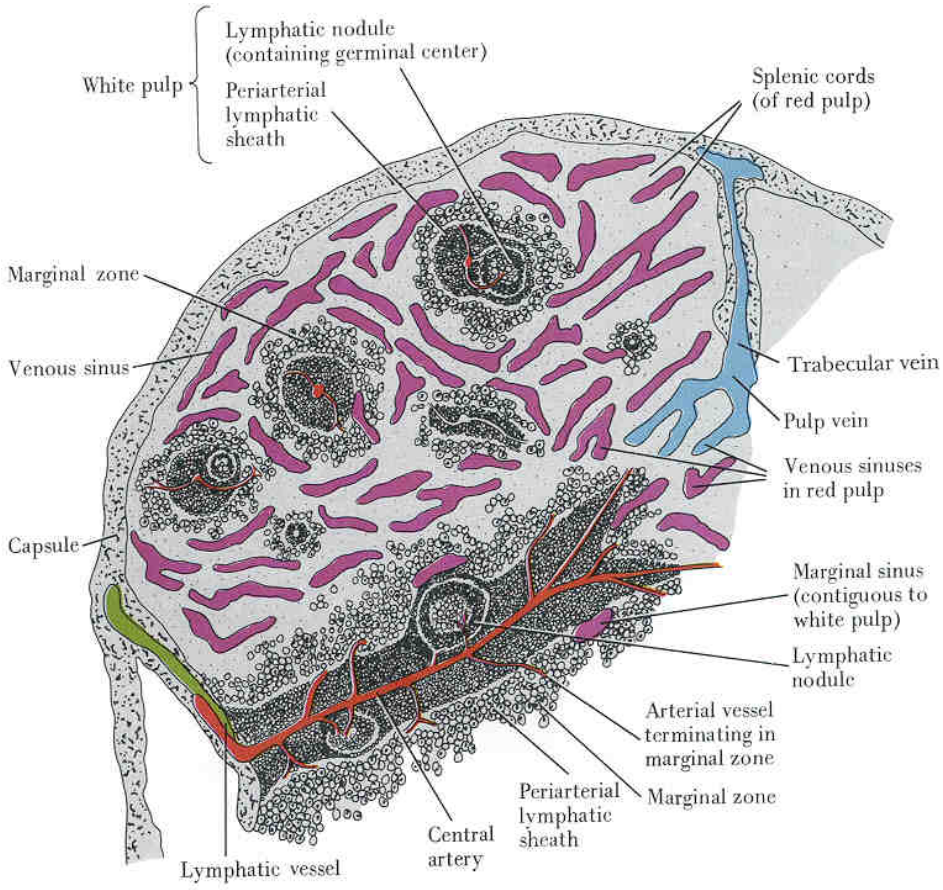

Figure 1.1 Structure of the spleen.

The bulk of the spleen is composed of the splenic pulp. Splenic pulp can be divided into white pulp, which consists of lymphoid tissue, and red pulp, which makes up the greatest mass of the spleen and is the site of red blood cells disposal. In addition, there are also specialized structures called splenic cords and splenic sinuses (or sinusoids). In a fresh histological section of the spleen the white pulp is seen as circular or elongated grey areas, surrounded by the red pulp.

The red pulp contains macrophages, lymphocytes, plasma cells and many additional blood elements (erythrocytes, granulocytes and platelets). Blood reaches the red pulp via branches of the central artery.

The white pulp consists of the lymphatic nodules with germinal centers and PALS (periarteriolar lymphoid sheath). Nodules are the territory of B lymphocytes, and the germinal center of a nodule is the site where B lymphocytes complete their maturation into plasma cells in response to antigenic challenge. In PALS T lymphocytes and DC are located.

The area surrounding the nodule is known as the marginal zone (MZ) and consists of a sinus lining reticular cells, marginal metallophilic macrophages (MM), marginal zone macrophages (MZM), marginal zone B cells and DCs (Weiss 1990;Aichele et al. 2003). The arterioles of the white pulp, derived from the central artery, empty into the sinuses of the marginal zone.

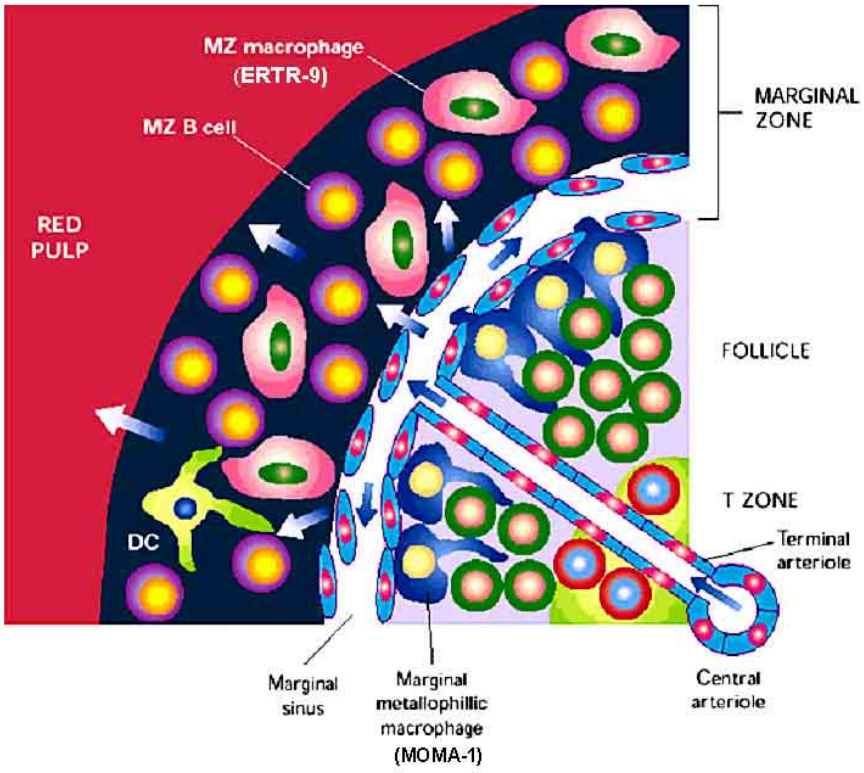

Figure 1.2 Marginal zone. Marginal zone is a unique lymphoid compartment positioned at a location where the bloodstream changes from a closed arterial circulation into the sinusoidal venous circulation of the red pulp. These areas contain different types of highly phagocytic macrophages, DCs and B cells and play important role in bacterial clearance (van der Laan et al. 1999).

MZM and MM are located at the strategic position where terminal arterioles open into the sinuses and the blood flow is reduced (Aichele et al. 2003). Additionally, they express a specific receptor repertoire, that allows them to trap antigens (Ags) like nanoparticles and fluoresceinated polysaccharides (Chao and MacPherson 1990). This area, thus, plays a significant role in filtering blood, where macrophages may phagocyte blood-borne antigen, or where antigen may contact the surface of dendritic reticular cells and B cells. Hence the marginal zone is crucial not only for Ag trapping and killing, but also is a crucial compartment for Ag presentation and the induction of the specific immune response (Aichele et al. 2003)

1.2 Innate immune reactions to infections

The host response to a foreign challenge usually requires the coordinated action of both the innate and acquired responses. Thus, adaptive immunity is only possible with the assistance of the innate immune system. Furthermore, the innate response provides a first line of defense of such efficiency, that invasion by most microorganisms does not result in infection. Such invasions regularly occur, as can be noticed in immunocompromised individuals. Consequently, pathogens are defined as microorganisms that have the ability to evade effector mechanisms of the innate immune system.

In addition to the activities already mentioned for cells of the innate immune system, like phagocytosis and killing of pathogens by production of effector molecules, an important property of the innate response is to recruit additional phagocytes to the sites of infection. This is mediated by releasing inflammatory mediators including many cytokines (Nelson and Krensky 1998) (Ulevitch and Tobias 1995).

This work is concentrated on reactions of the innate immune system induced by *Listeria monocytogenes*; therefore the activities of this system, described here, will be biased towards reactions raised by this bacterium.

1.2.1 Receptors

Depending on the particular infective microorganism, cells of the innate immune system usually respond in the appropriate way, i.e. by activation the proper effector cells. Responsible for this is a panel of receptors, so called opsonin independent pattern recognition receptors, like Toll-like receptors, scavenger receptors, lectins and CD14 (Peiser et al. 2002), but also receptors binding to opsonins. Interestingly receptors binding to opsonins can also act in an opsonin independent manner e.g. complement receptor 3 (CR3)(Ofek et al. 1995) (Ishibashi et al. 1994).

1.2.1.1 Pattern recognition receptors

Opsonin independent receptors often do not just bind to a single ligand, but rather recognize particular types of Pathogen-Associated Molecular Patterns (PAMPs) on the surfaces of

microorganisms. These receptors are called pattern recognition receptors (PRRs) and they can be both, soluble and cellular (Kurt-Jones et al. 2000).

Toll-like receptors

Macrophages, dendritic cells, and epithelial cells have a set of transmembrane receptors called Toll-like receptors (TLRs). They are named TLR due to their homology to the TOLL receptor first discovered and named in *Drosophila melanogaster*, essential in the embryonic development (Lien et al. 2000) and in the immune response to microbial infection in this species (Tauszig et al. 2000) (Hoffmann and Reichhart J.M. 2002).

To date 10 different TLRs are known (Ozinsky et al. 2000;Akira 2003):

- TLR1 – as heterodimer with TLR2, binds lipoprotein
- TLR2 – as a homodimer recognizes LTA, as a heterodimer with other TLR family members (e.g. TLR1 and TLR6), binds various fungal, Gram-positive, and mycobacterial components like peptydoglikan, lipoprotein or LPS
- TLR3 – specific for poly I:C and double stranded RNA
- TLR4 – recognizes LPS (together with TLR4-accessory molecule MD-2 and CD14)
- TLR5 – binds to bacterial flagellin
- TLR6 – as heterodimer with TLR2 binds lipoprotein as well as MALP-2 from *Mycoplasma*
- TLR7 – specific for imidazoquinolines (antivirus compounds)
- TLR8 – specific for imidazoquinolines (only human TLR8)
- TLR9 – recognizes unmethylated CpG containing DNA motifs
- TLR10 – unknown specificity

Activation via TLRs stimulates cells of the innate immune system involving adaptor molecules, like MyD88 (Fitzgerald et al. 2001) or TIRAP (Horng et al. 2001), kinases (IRAK) and transcriptional factors. TLRs activation always includes the activation of NF-κB. NF-κB, a key transcription factor that mediates early host defenses (Luster 2002), is involved in the induction of the genetic programs that are essential for host defense, including the induction

of cytokines and costimulatory molecules (Akira et al. 2001;Edelson and Unanue 2002;Luster 2002).

Activated TLR determines the type of response to a particular pathogen. In this way, the TLRs identify the nature of the pathogen and trigger an effector response appropriate for dealing with it. Interestingly, in macrophages and dendritic cells, the TLRs are exposed to the pathogen inside the phagosome (Ozinsky et al. 2000).

Especially important for listerial recognition are TLR2 as a heterodimer with TLR6 (specific for peptydoglican), TLR2 as a homodimer (lipoteichoic acid) (Toshchakov et al. 2002;Underhill and Ozinsky 2002), TLR5 (flagellin)(Hayashi et al. 2001) and TLR9 (bacterial DNA) (Hemmi et al. 2000;Edelson and Unanue 2002).

Scavenger receptors

Another type of receptors involved in innate responses are scavenger receptors (SRs). These receptors, first time described in 1979 (Goldstein et al. 1979), are expressed on myeloid cells (macrophages and dendritic cells, but not monocytes or neutrophils) and certain endothelial cells. SRs encompass a broad range of molecules involved in receptor-mediated endocytosis of selected polyanionic ligands (Greaves et al. 1998;Peiser et al. 2002). Additional ligands include proteins, polyribonucleotides, polysaccharides and lipids, but also microorganisms and their products like Gram-positive bacteria (lipoteichoic acid), Gram-negative bacteria (LPS) and CpG DNA (Peiser et al. 2002) (Peiser and Gordon 2001)

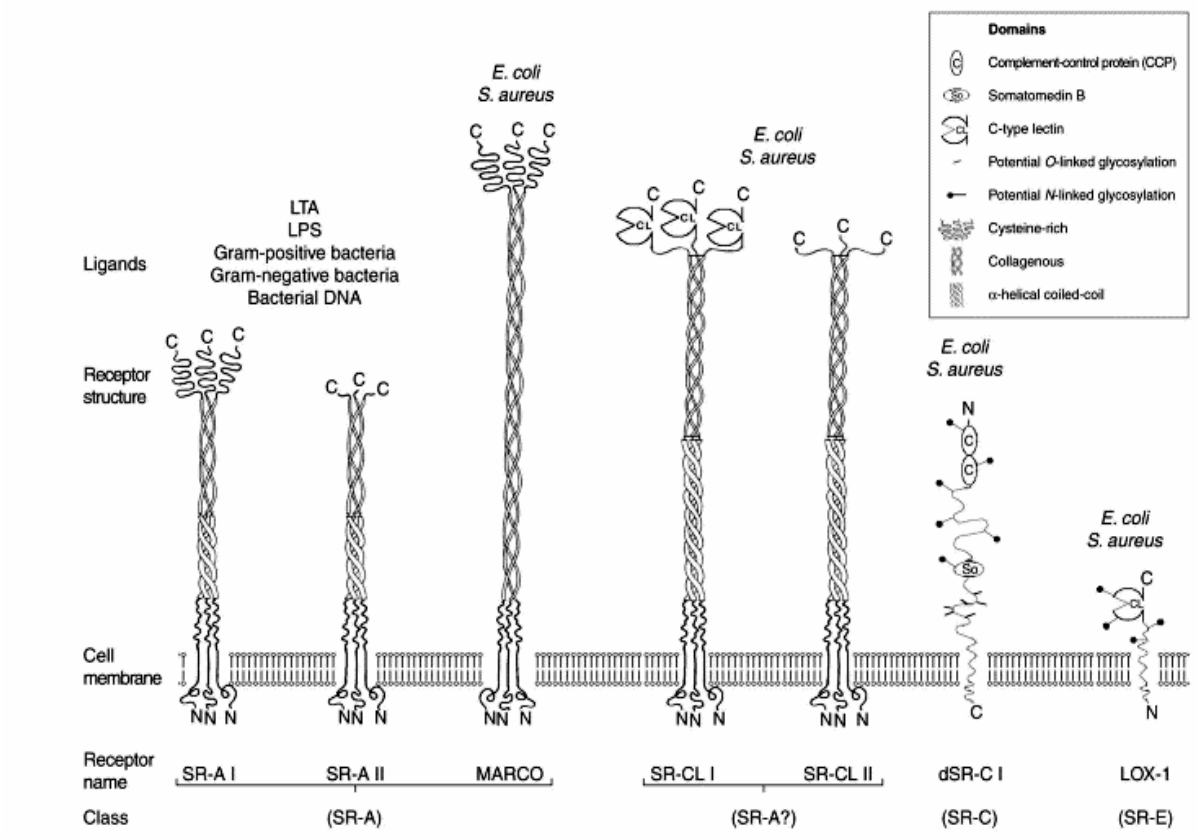

Figure 1.3 Scavenger receptors (Peiser et al. 2000)

SRs may have diverse functions:

- endocytosis, thus increasing antigen presentation to T cells (Abraham et al. 1995)
- cell-cell adhesion (van Velzen et al. 1999) and surface adhesion e.g. tissue culture plastic (Fraser et al. 1993)
- phagocytosis of bacteria (Thomas et al. 2000;Peiser et al. 2000) and pattern recognition
- uptake of apoptotic cells (Savill and Fadok 2000)

The structural basis for receptor-ligand interactions remains unclear (Gordon 2002). The binding site for selected polyanionic ligands has been attributed to the collagenous domain. An additional coiled-coil domain appears to be important for receptor trimerisation. The cytoplasmic tail has no known internalization motifs, but contains potential protein kinase C (PKC)-interaction sites (Figure 1.3).

SRs on macrophages belong to the SR-A family, consisting of SR-A I, SR-A II and MARCO (Peiser et al. 2002).

MARCO (macrophage receptor with a collagenous structure) is a distinct type-A SR, containing collagenous and Scavenger Receptors Cysteine Rich (SRCR) domains, but is very similar to SR-A I/II overall (Elomaa et al. 1995). The mouse molecule is able to bind Gram-positive and Gram-negative bacteria, but is normally expressed by only a subpopulation of MØ—for example in spleen marginal zone and freshly harvested peritoneal populations. It is rapidly induced in most tissue MØ by bacterial infection or treatment with bacteria or LPS *in vitro* (Kraal et al. 2000)

Importantly, recent studies showed that SR-AI/II knockout mice have a higher susceptibility to infection with *Listeria* (Suzuki et al. 1997).

How these receptors signal is not very well understood.

Lectins

Another family of receptors expressed by macrophages, DCs, and some endothelial cells are lectins, simply defined as proteins that specifically bind (or crosslink) carbohydrates. Their ligands are present on a range of bacteria, fungi, virus-infected cells, and parasites. Since lectins generally have no apparent catalytic activity like enzymes, their physiological functions remain unclear. Lectins can functionally be distinguished by whether they recognize endogenous or exogenous ligands. Lectins that recognize endogenous ligands appear to play a role in fertilization and development, and their function often involves cell-to-cell adhesion (Arason 1996).

Lectins recognizing exogenous ligands are divided into two main groups:

- C-type; able to recognize mannose groups on bacteria and mediate the destruction of these pathogenic invaders through the activation of complement (Mannose-binding protein (MBP)) (Nakagawa et al. 2003) or mediate the initial adhesion of moving leukocytes to the endothelium during rolling - essential for leukocyte recruitment into inflamed tissue (Selectins)(Graves et al. 1994)
- S-type; galectins are small, soluble proteins. These receptors modulate cell-cell adhesion (Lobsanov et al. 1993) and might regulate cell growth (Yang et al. 1996)

The downstream signaling of lectins is as yet not well established.

CR3 receptor

CR3 is a myeloid cell phagocytic receptor for particles opsonized by complement, irrespective of host or microbial origin, but also for direct interactions with pathogens such as *Mycobacterium tuberculosis* and yeast-derived zymosan as well as host ligands (Ross 2000). CR3 is a β2 integrin also known as CD18/CD11b (Mac-1), and plays a key role in myelomonocytic cell recruitment to sites of inflammation. Its expression by tissue macrophages is selective, e.g., by microglia, but not resident alveolar macrophages and binds a diverse range of ligands, including ICAM-1, selected clotting components, senescent platelets, and possibly, denatured proteins. CR3 contributes to the clearance of apoptotic cells and may provide a relatively "silent" means of entry to macrophages for selected pathogens. The opsonic phagocytic mechanism differs from that mediated by Fc receptors, and CR3-mediated uptake by macrophages does not trigger release of arachidonate or reactive oxygen metabolites. Structural studies have proven difficult, and the signaling pathways involved remain poorly defined (Gordon 2002).

1.2.1.2 Opsonin-dependent receptors

In opsonin-dependent phagocytosis, antibodies or complement molecules bind to microorganisms, thereby promoting ingestion via Fcγ or complement receptors on phagocytic leukocytes.

Fcγ receptor

Stimulation of monocytes through the Fcγ receptors can activate a variety of responses, including phagocytosis, production of cytokines, and release of reactive oxygen intermediates (Greenberg et al. 1993). Three classes of Fcγ receptors have been found on human monocytes. FcγRI and FcγRII are expressed constitutively, but at varying levels; FcγRIII expression is low but is induced upon differentiation of monocytes. FcγRI has a high affinity for monomeric IgG, whereas FcγRII and FcγRIII bind preferentially to aggregated IgG. FcγRIII occurs in two distinct forms; the one found in monocytes is a single polypeptide chain with extracellular, transmembrane and cytoplasmic domains. The other form, found in neutrophils, occurs as an oligomeric complex of a phosphatidylinositol anchored chain (α) together with dimers of an intracellular chain (γ or ζ). The initial signaling events downstream of the Fc receptor appear similar to those downstream of the TCR (Weiss and Littman 1994).

FcεR is another Fc receptor described. It is a high affinity receptor of the immunoglobulin superfamily that binds IgE on mast cells, basophils and activated eosinophils and is especially important in allergic responses (Ravetch and Kinet 1991).

C1q complement receptor

The primary function for C1q has been its participation in the effector arm of the humoral immune response as the recognition subunit of C1, the first component of the classical complement cascade (CCP) (Cooper 1985). However, although immune complexes are often considered the major activator of C1 via the binding of C1q to antibody Fc domains, it is known that C1q is able to bind directly to various substances, including certain viruses and bacteria, and activate CCP in an Ab-independent manner. In addition, C1q has been shown to interact with cells of the immune system, including monocytes, neutrophils, B cells, and eosinophils, as well as with endothelial cells (Bobak et al. 1986). It initiates a variety of cellular responses, including the stimulation of different host defense mechanisms, depending on the cell type to which it binds. Specifically, C1q enhances both FcR- and CR1-mediated phagocytosis by human monocytes and macrophages *in vitro*, phagocytosis of immune complexes by human neutrophils and also triggers the generation of toxic oxygen species such as superoxide anions by human neutrophils (Ohkuro et al. 1994). C1q has also been shown to enhance killing of the microorganisms by neutrophils (Nepomuceno and Tenner 1998).

Listeria monocytogenes can induce its own uptake via CR1q, which is recognized by InlB on the bacterial surface (Cossart et al. 2003).

C3 complement receptor

C3 receptor is described in more detail in point 1.2.1.1. Many pathogens can activate this receptor spontaneously and are opsonized this way.

It is known that *L. monocytogenes* phagocytosed by macrophages via the CR3 receptor are readily killed in the absence of inflammatory mediators (Drevets et al. 1993)

1.2.2 Reactions elicited by receptor triggering

As mentioned before the combination of receptors triggered by a particular microorganism results in differential reactions of infected phagocytes. In general, reactions include

upregulation of antigen presenting capacity, upregulation of cell adhesion molecules and co-stimulatory molecules, induction of low molecular weight effector molecules and induction of cytokines.

1.2.2.1 Antigen presentation

T cells cannot recognize antigen directly. Therefore, the antigen needs to be processed (proteolyticaly degraded) and presented on the cell surface bound to molecules encoded by the Major Histocompatibility Complex (MHC). Complex mechanisms are involved in this process and the molecules involved are usually upregulated during infections, resulting in an increased ability to stimulate T cells. T cell stimulation is particularly dependent on DCs, since they carry the Ags to the secondary lymphoid organs and are the only APCs that are able to activate naïve resting T cells (Pron et al. 2001).

1.2.2.2 Cell adhesion and co-stimulatory molecules

The first encounter between T cells and APCs is unspecific due to cell adhesion molecules. Upregulation of such molecules during infection increases the capacity to stimulate T cells (Ardavin 2003). In addition, activation of T cells via TCR requires costimulatory molecules like B7.1 and B7.2 that simultaneously provide essential signals via CD28 for the activation of T cells. Upregulation of such costimulants is often the result of an infection, thus, again increasing the capacity of infected APC to trigger T cells (Maldonado-Lopez et al. 1999).

1.2.2.3 Low molecular weight mediators

As already mentioned, infection triggers an induction of low molecular weight effector molecules, like free radicals or lipid metabolites in neutrophils, macrophages, endothelial and other cells. There are two types of free radicals: reactive oxygen intermediates (ROI) which are created in neutrophils by the activity of NADPH oxidase, and the reactive nitrogen intermediates (RNI) produced in reaction catalyzed by nitric oxide synthase (iNOS). These mediators are induced by stimulation of particular enzymatic pathways leading to substrate reduction or oxidation.

Prostaglandins (PGs) and leukotrienes (LTs) are locally secreted, pro-inflammatory lipid mediators resulting from metabolic degradation of the arachidonic acid by phospholipase A_2. Arachidonic acids originate from membrane phospholipids (Murphy and Hankin 1999). LTs are of importance in host defense reactions and have a pathophysioloigcal role in inflammation and allergic reactions. PGs have a wide variety of actions but most cause inflammation, cell proliferation, differentiation and apoptosis.

1.2.2.4 Cytokines

Cytokines induced during infection are the class of mediators most relevant in this work. This group consists of simple polypeptides or glycoproteins, about 30 KDa in size. Many of them form functional homo- or heterodimers. Their constitutive production is usually low, and they are transiently upregulated upon stimulation. Cytokines act by binding to a high affinity cell surface receptors and trigger signaling pathways that lead to changes in gene expression. The most characteristic feature of cytokines that distinguish them from hormones, is their redundancy and pleiotropy, i.e. structurally dissimilar cytokines can be similar in their action e.g. IL-1 (Interleukin-1) and TNF-α (Tumor Necrosis Factor α). In addition they exert a multitude of actions on different cells and tissues. Under natural conditions, cells rarely encounter only one cytokine at a time; rather it is a cocktail of several cytokines, resulting in biological actions reflecting synergistic and antagonistic interactions between those agents.

For instance, the secretion of IL-1, IL-6 and TNF-α results in (Biron 1998) (Springer 1994):

- release of neutrophils from the bone marrow.
- alteration of the body temperature by acting on hypothalamus
- energy mobilization by degradation of the proteins in muscle or fat cells
- induction of acute phase proteins in the liver
- induction of DCs maturation and their migration into lymph nodes
- upregulation of Ag presentation and costimulation

Thus, it is obvious that the release of several cytokines has a pleiotropic effect for the whole body.

Pro-inflammatory cytokines

Inflammatory cytokines produced by phagocytes consist of IL-1, IL-6, IL-12 and TNF-α among others. Secretion of pro-inflammatory cytokines results in the activation of several mechanisms, e.g. induction of acute phase response or leukocyte migration.

IL-1

IL-1 has a very wide range of biological activities on different target cells. *In vivo* it induces hypotension, fever, weight loss and neutrophilia. IL-1 is required for resistance to listeriosis *in vivo* (Rogers et al. 1992;Havell et al. 1992;Rogers et al. 1994). Neutralization of IL-1 results in decreased numbers of neutrophils in infective foci and macrophage unresponsiveness to IFNγ (Unanue 1997)

IL-6

IL-6 is secreted by both lymphoid and non-lymphoid cells and regulates B and T cell function, haematopoiesis and acute phase responses (Kishimoto et al. 1992). Lack of IL-6 seems to increase susceptibility of mice to listeriosis (Kopf et al. 1994;Miura et al. 2000), however its importance in the defense against *Listeria* is controversial (Barsig et al. 1998).

IL-12

IL-12 is an important molecule in the defense against intracellular pathogens (Locksley 1993). It induces IFNγ production by T and NK cells, enhances NK activity and stimulates proliferation and differentiation of the Th1 subset of T cells (Hsieh et al. 1993;Manetti et al. 1993). *In vitro*, IL-12 is produced by activated macrophages (Tripp et al. 1993), DC (Heufler et al. 1996) and neutrophils (Cassatella et al. 1995). This cytokine has been shown to be essential for mice resistance to *Listeria* as neutralizing IL-12 with antibodies (Abs) results in poor macrophage activation and an increase of bacterial growth in liver and spleen.

TNFα

TNFα is a potent paracrine mediator of inflammatory and immune functions. It regulates growth and differentiation of a wide variety of cells types. TNFα together with IL-12 has been shown to be essential for NK cell activation and subsequent IFNγ production (Wherry et

al. 1991;Tripp et al. 1993). Administration *in vivo* of anti-TNFα Abs leads to inhibition of macrophage activation during *Listeria* infection (Pfeffer et al. 1993).

Interferons

Interferons were originally described as inflammatory mediators acting against virus infections (Isaacs and Lindenmann 1957). However, lately they are rather recognized as important regulatory molecules in the immune system as well as in ontogeny (Inaba et al. 1986). This group can be further divided into type I and type II interferons.

Type I interferons (IFN α/β).

Molecules of this family of cytokines, produced upon virus and intracellular organisms infection, play essential role in conferring cells an antiviral state (Pestka et al. 1987;Taki 2002).

The group of cytokines called IFN α/β consist of (Soos and Szente 2003;Kotenko et al. 2003)

- IFN-α (a family of ~ 18 non-allelic genes, closely related proteins) (Soos and Szente 2003)
- IFN-β (product of a single gene)(Sheppard et al. 2003)
- IFN-ω (formerly IFNα -II, at least 5 genes) murine equivalent unknown (Pestka et al. 1987)
- IFN-τ (product of a single gene) only in ruminant ungulate species (Roberts et al. 1999)
- IFN-κ (LaFleur et al. 2001)
- IFN λ (IL-27 and IL-28) (Kotenko et al. 2003)
- IFN-ε (Conclin, DC, Grant, F.J., Rixon, M.W.& Kindsvogel, W. Interferon-ε. US patent 6329175 (2002))
- Limitin (Takahashi et al. 2001)

Type I interferons show at least 30% sequence homology and are encoded in a region of 4 of murine chromosome (De Maeyer and De Maeyer-Guignard 1988). They have also similar biological activities and bind to a single heterodimeric receptor called IFNα/β receptor

(except for IFN λ). IFNα/β R activates signal transduction pathways mainly via the Jak kinases, Jak1, Tyk2 and Stat1 or Stat2 (Stark et al. 1998;Takahashi et al. 2001;Taki 2002).

In addition to the direct antiviral function, they also have a role in the regulation of the immune system (Gresser I 1990). They induce MHC class I, a number of cytokines (IL-10, IL-15 in macrophages) (Durbin et al. 2000), chemokines and inducible nitric oxide synthase (iNOS) (Taki 2002). They also activate antiviral NK cells (Biron et al. 1999), DCs (Luft et al. 1998), naïve $CD8^+$ cells (Sun et al. 1998) and drive human $CD4^+$ T cells differentiation into a Th1 direction (Wenner et al. 1996;Stockinger et al. 2002). Recently IFNβ has been shown to influence the bone structure (Takayanagi, 2002 Nature: 744) and to take part in stress response (Ortiz et al. 2003)

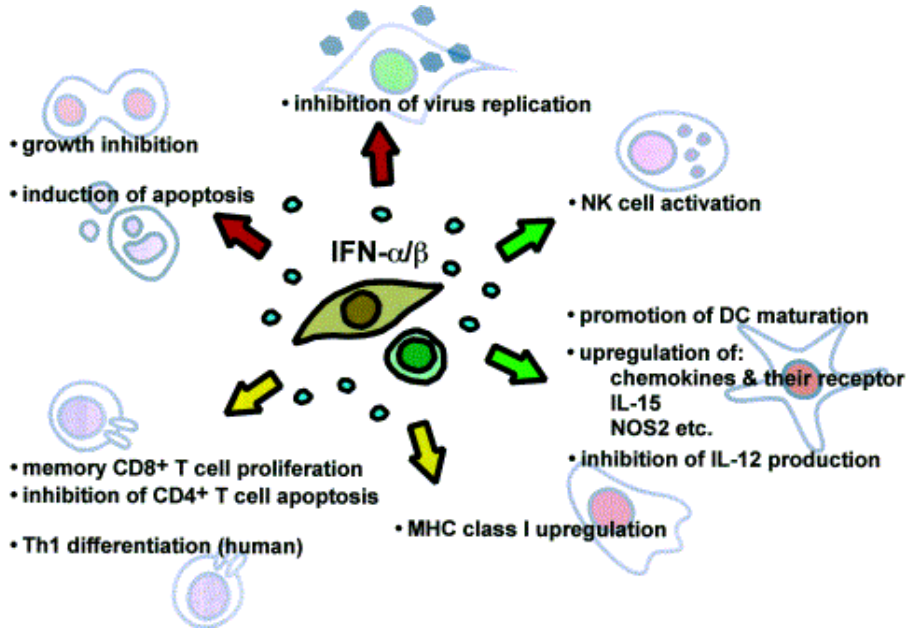

Figure 1.4 Multiple biological activities of IFN α/β. (Taki 2002).

Susceptibility to *Listeria monocytogenes* infection in IFN α/β receptor knockout is not altered (Muller et al. 1994), however, type I interferons seem to decrease the speed and efficacy of clearing *Listeria* infection (Stockinger et al. 2002).

Type II interferon (immune interferon)

IFNγ is synthesized mainly by NK cells and effector T cells (Farrar and Schreiber 1993;Boehm et al. 1997). It is the product of a single gene on the murine chromosome 10. It

has a pleiotropic effect on immune and inflammatory responses, e.g. activation, growth and differentiation of T cells, macrophages and NK cells. IFNγ stimulates macrophages to kill bacteria that have been engulfed. Antiviral activity is rather weak in comparison to the Type I interferons. The IFNγ released by Th1 cells is also important in regulating the Th1/Th2 response, by induction of IL-12 production in macrophages. IFNγ represents a major cytokine of the adaptive immune response, but recently APCs of the myeloid lineage were also found to secrete IFNγ (Frucht et al. 2001).

Mice pretreated with neutralizing Abs against IFNγ lose the ability to resist a sublethal dose of *L. monocytogenes* (Buchmeier and Schreiber 1985) (Bancroft et al. 1989). Furthermore mice with targeted mutations in the genes encoding IFNγ, IFNGR1, IFNGR2 (IFNγ receptors) and Stat1 die during the challenge with sublethal dose of this bacterium (Dalton et al. 1993) (Huang et al. 1993;Meraz et al. 1996;Lu et al. 1998).

Chemokines

Chemokines are low molecular weights (8-12 kDa) cytokines, which selectively attract and activate distinct types of leukocytes (Baggiolini 1998). They are produced by several cell types and are considered important mediators of inflammation. Induced during viral, bacterial and protozoal infections, chemokines have been shown to be tightly controlled by cytokines. IL-10 for example elicits CCL2 release by unstimulated human blood mononuclear cells (Seitz et al. 1995;Yano et al. 1996). IL-4 increases the level of CCL2 in human endothelial cells (Rollins and Pober 1991;Yano et al. 1996). TNFα, IL-1 and IFNγ stimulate release of CCL2, CCL11 and CCL13 in human endothelial cells (Proost et al. 1996) (Garcia-Zepeda et al. 1996).

Chemokines share common signaling pathways, which include intracellular calcium mobilization (Imai 1997) (Combadiere et al. 1998;Maciejewski-Lenoir et al. 1999), MAP kinase (Maciejewski-Lenoir et al. 1999;Kansra et al. 2001;Cambien et al. 2001b), tyrosine kinase (Cambien et al. 2001a) and PI-3K (Kansra et al. 2001;Cambien et al. 2001a) activation.

Based on expression level and function, chemokines can be divided into two groups: inflammatory inducible chemokines that recruit leukocytes in response to physiological stress, and homeostatic constitutive chemokines responsible for basal leukocyte trafficking and forming the architecture of the secondary lymphoid organs (Cyster 1999) (Moser and

Loetscher 2003). The distinction between these two groups is not absolute; several chemokines need to be included in both subfamilies (Moser and Loetscher 2003).

Therefore chemokines are also divided into different families depending on the presence and arrangement of the cysteines in highly conserved N-terminal positions – C, CC, CXC, CX_3C. CC, CXC and CX_3C chemokines have four cysteines, where the two N-terminal are intercalated by 0, 1 and 3 undefined amino acids, respectively. A single C chemokine contains only two cysteines – corresponding to the second and fourth cysteines of the other groups (Figure 1.5).

The nomenclature of chemokines has now been standardized since several names often were assigned to the same molecule. Now they are called according to the chemokine group membership, similarly their receptors are now designated (Table 1.1).

C family (γchemokine)

XCL1 (lymphotactin) is the only member of this family. It is unique among chemokines as it contains only one of the two disulfide bridges that are conserved in all other chemokines and possesses a unique C-terminal extension, required for biological activity (Kelner et al. 1994; Hedrick and Zlotnik 1997;Marcaurelle et al. 2001;Kuloglu et al. 2002). Lymphotactin exerts a chemotactic activity mainly on T and NK cells, but has recently been found to attract also neutrophils and B cells through the XCR1 receptor (Yoshida et al. 1998;Cairns et al. 2001).

CC family (β chemokines)

The first two cysteine residues are adjacent. CC chemokines act on a diverse spectrum of target cells (Rollins 1997), but preferentially on monocytes and lymphocytes. The first β chemokine cloned CCL2 (Monocyte Chemoattractant Protein; MCP-1) is the most potent chemoattractant for monocytes, not including neutrophils. It induces the expression of integrins required for chemotaxis (Vaddi and Newton 1994) and also attracts CD4 and CD8 memory T lymphocytes and NK cells. Other MCPs like CCL8, CCL7, CCL13 and CCL12 (MCP-2, 3, 4 and 5, respectively) have similar functions but lower efficiency (Rollins 1997).

CCL3 and CCL4. (Macrophage Inflammatory Proteins-1 α and β, respectively) also attract and activate monocytes but much less efficiently than MCPs (Rollins 1997) also $CD4^+$, $CD8^+$, NK cells and DCs.

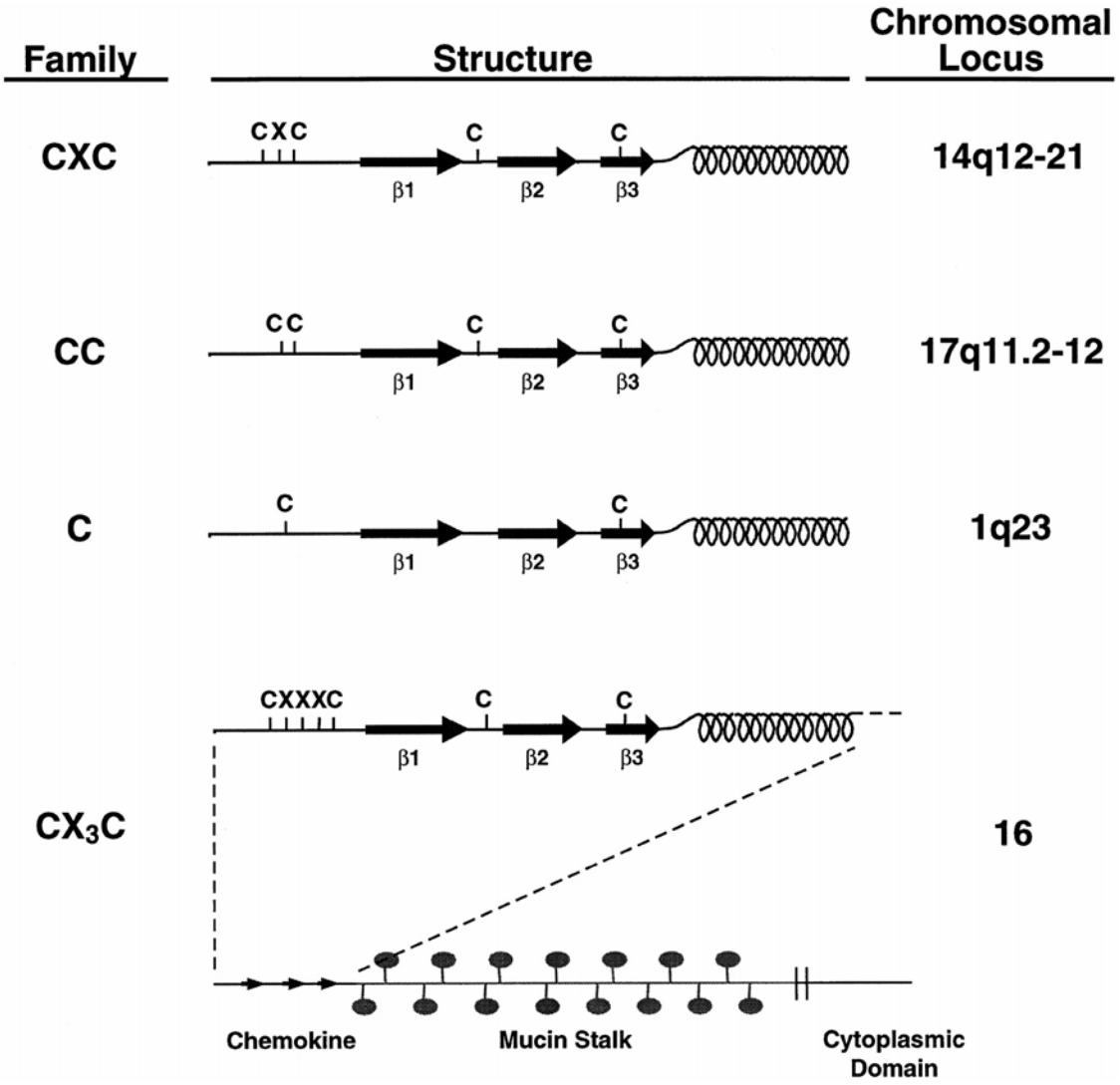

Figure 1.5 Chemokine families. Chemokines are divided into families based on structural and genetic considerations. All chemokines are structurally similar, having at least three β-pleated sheets (indicated as b1-3) and a C-terminal α-helix. Most chemokines also have at least four cysteines in conserved positions. In the CXC chemokine family, the two cysteines nearest the N-termini of family members are separated by a single (and variable) amino acid. The genes encoding these proteins cluster at human chromosome 14q12-21 (except for SDF-1α whose gene maps to chromosome 1053). In the CC chemokine family, the two cysteines nearest the N-termini of these proteins are djacent. Their genes cluster at 17q11.2-12 (except for MIP-3β, whose gene maps to chromosome 9117 and MIP-3α/LARC which maps to chromosome 2117a). Lymphotactin is a structurally related chemokine having only one cysteine near ist N-terminus and is said to belong to the C chemokine family. The CX3C chemokine (also called ‘fractalkine’’ or ‘‘neurotactin’’) has a typical chemokine-like structure at its N-terminus except for the placement of three amino acids between the first two cysteines. This chemokine domain occurs at the end of a long stalk which is heavily substituted with mucin-like carbohydrates. The protein is embedded in the membrane and has a short cytoplasmic domain. (Rollins 1997).

CCL5 (RANTES; Regulated upon Activation, Normal T cell Expressed and Secreted) – is a chemoattractant of monocytes, eosinophils, $CD4^+$ and $CD8^+$ T cells. For $CD8^+$ cells it represents the most potent attractant among CC chemokines (Rollins 1997).

CXC family (α chemokines)

The first two cysteines in CXC chemokines are separated by a single amino acid. They are secreted molecules, except CXCL16, which is a membrane-bound molecule. CXCL chemokines can be further divided into a two subclasses, based on the presence or absence of tripeptide motif glutamic acid-leucine-arginine (ELR) N-terminal to the first cysteine. This classification is correlated with the functional differences (Rollins 1997):

- ELR-containing CXC chemokines – potent chemoattractants and activators of neutrophils, like CXCL1, CXCL2 and CXCL3 (GROα, β and γ) (Ahuja and Murphy 1996)
- Non-ELR CXC chemokines – specific first of all for T-cells, tumor-infiltrating lymphocytes and mononuclear cells, like CXCL9, CXCL10 and CXCL12 (MIG, IP-10 and SDF-1)

CX_3C family

At N-terminus of this protein two cysteines are separated by three amino acids. CX_3CL1 (fractalkine) represents the sole member of this subfamily. This chemokine is structurally unique. Functionally it is a membrane associated protein and acts like an adhesion molecule (Goda et al. 2000). It is principally localized on DCs, endothelial and epithelial cells, and upregulated under inflammatory conditions. Endothelial cells upregulate this chemokine after stimulation with inflammatory cytokines like TNFα, IL-1 and IFNγ (Bazan et al. 1997) (Harrison et al. 1999;Garcia et al. 2000).

Increased expression of this chemokine on the surface of maturating DCs suggest that CX_3CL1 could be involved in DC-lymphocyte interactions (Papadopoulos et al. 1999).

This chemokine is also constitutively expressed by neurons in CNS (Central Nervous System) (Pan et al. 1997;Harrison et al. 1998;Nishiyori et al. 1998).

Systematic Name	Human Common Name	Mouse Common Name	Receptors Bound	Expression	Proposed Function and Source
CXCL1	GROα, MGSA	MIP-2, KC	CXCR2	Inducible	Neutrophilic inflammatory sites
CXCL2	GROβ, MIP-2α	KC	CXCR2	Inducible	Neutrophil chemotaxis; lesions
CXCL3	GROγ, MIP-2β	KC	CXCR2	Inducible	Neutrophil chemotaxis
CXCL4	PF4	PF4			
CXCL5	ENA-78	LIX	CXCR2	Inducible	Neutrophilic inflammatory sites
CXCL6	GCP-2	CKα-3	CXCR1,2	Inducible	Neutrophilic inflammatory sites
CXCL7	NAP-2		CXCR2	Inducible	Neutrophilic inflammatory sites
CXCL8	IL-8		CXCR1,2	Inducible	Neutrophilic inflammation; liver, acute lung injury; atherosclerotic lesions
CXCL9	Mig	Mig	CXCR3	Inducible	Th1 inflammation, monocyte
CXCL10	IP-10	IP-10, CRG-2	CXCR3	Inducible	chemotaxis; CNS, intestinal lesions
CXCL11	I-TAC		CXCR3	Inducible	Th1 inflammation
CXCL12	SDF-1	SDF-1	CXCR4	Constitutive	T cells activation, bone marrow; thymus; lung; lymphoid organs
CXCL13	BLC, BCA-1	BLC, BCA-1	CXCR5	Constitutive	Lymphoid follicles
CXCL14	BRAK, bolekine				
CXCL15		lungkine			
CXCL16	CXCL16	CXCL16	CXCR6		Th1 inflammation
CCL1	I-309	TCA-3	CCR8	Inducible	Th2 inflammation
CCL2	MCP-1, MCAF	JE	CCR2	Inducible	Monocytes chemotaxis and activation,Th1 inflammation, NK cells chemotaxis
CCL3	MIP-1α	MIP-1α	CCR1,5	Inducible	Monocytes chemotaxis, Th1
CCL4	MIP-1β	MIP-1β	CCR5,8	Inducible	inflammation;
CCL5	RANTES	RANTES	CCR1,3,5	Inducible	Th1, Th2 inflammation; Lung, CNS, skin injury; atherosclerotic lesions
CCL6		MRP-1, C10			Bone marrow cells, macrophages
CCL7	MCP-3	MARC	CCR1,2,3	Inducible	Monocytes chemotaxis and activation, Th1, Th2 inflammation
CCL8	MCP-2	MCP-2	CCR3	Inducible	
CCL9		MRP-2, MIP-1γ			
CCL10		CCF18			
CCL11	eotaxin	eotaxin	CCR3	Inducible	Th2 inflammation; allergic lung, skin disease
CCL12		MCP-5	CCR2	Inducible	Th1, Th2 inflammation; allergic lung
CCL13	MCP-4		CCR2,3	Inducible	disease
CCL14	HCC-1, CKβ1		CCR1	Constitutive	Monocytes chemotaxis; plasma, spleen, liver
CCL15	HCC-2, Lkn-1, MIP-5		CCR1,3	Constitutive	
CCL16	HCC-4, LEC, Mtn-1	LCC-1	CCR1, CCR2	Constitutive	
CCL17	TARC	TARC	CCR4	Both	Th2 inflammation in skin
CCL18	DC-CK1, PARC			Constitutive	Lymphoid T cell zones
CCL19	MIP-3β, ELC, ckβ11	MIP-3β, ELC	CCR7	Both	Lymphoid T cell zones
CCL20	MIP-3α, LARC	MIP-3α, LARC	CCR6	Both	Intestinal villi; skin
CCL21	6Ckine, SLC, ckβ9	SLC, TCA-4	CCR7	Constitutive	Lymphoid organs, HEV
CCL22	MDC, STCP1	abcd-1	CCR4	Both	Thymus; allergic lung disease; Th2 inflammation
CCL23	MPIF-1, ckβ8-1		CCR1	Inducible	
CCL24	MPIF-2, eotaxin-2		CCR3, CCR5	Inducible	Th2 inflammation
CCL25	TECK, ckβ15	TECK, ckβ15	CCR9	Constitutive	Small intestine; thymus
CCL26	eotaxin-3, MIP-4α		CCR3	Inducible	Th2 inflammation
CCL27	CTACK, ILC, ESkine	ALP, skinkine	CCR10	Constitutive	Skin
CX_3CL1	fractalkine	neurotactin, NTN	CX_3CR1	Both	Lymfocytes; CNS. periphery
XCL1	lymphotactin, ATAC	lymphotactin	XCR1	Inducible	T cells, bone marrow cells chemotaxis

Table 1.1 Characteristics of chemokines (Olson and Ley K 2002), (Mantovani et al. 2003), (Mukaida et al. 2003).

Chemokines exert their attractant effect only when expressed locally at low levels and systemically administered chemokines antagonize the local effects (Rollins 1997). They also appear to be able to recruit leukocytes without necessarily activating them.

Macrophages are one of the sources of chemokines and their expression is controlled by a complex network of signals. Apart from a direct effect of the microorganism during acute infections, the activation of macrophages is controlled by pro-inflammatory cytokines as activating factors, and by anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 as deactivating factors (Locati and Murphy 1999;Mantovani et al. 2003).

Chemokines receptors

One problem in understanding chemokine physiology is the fact that many chemokines share the same receptors, and can bind to several other receptors. The complexity of induced chemokines becomes obvious from Table 1. Receptors are important not only for leukocyte migration and the control of inflammatory responses, but also for an architecture of the secondary lymphoid tissues (Rollins 1997). All chemokine receptors belong to the family of the 7-transmembrane spanning G protein-coupled receptors and their activation leads to the different signal transduction pathways through PI3 kinase, MAP kinase or cAMP. Clearly other signal transduction pathways are involved, but mechanisms coupling receptor triggering with the complex physiological responses are still being investigated (Rollins 1997).

1.3 *Listeria monocytogenes*

Listeria monocytogenes, a gram-positive facultative intracellular bacterium of low G+C content, was discovered in 1924, by E.G.D. Murray, R.A. Webb and M.R.B. Swan as the etiological agent of septicemia in rabbits (Murray E.G.D. et al. 1926).

The genus *Listeria*, facultative anaerobic rods, isolated from a variety of foods, soil, water and animal feces, is closely related to the genus *Bacillus*, *Clostridium* and *Staphylococcus*. The whole genus includes six species – *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi* but only the first two are potentially pathogenic, causing listeriosis (Vazquez-Boland et al. 2001).

L. monocytogenes causes serious localized and systemic infections in mammals and birds, and is associated with serious infections in immunocompromised individuals, pregnant women

and elderly. Listeriosis is a food-borne infection. Mostly the source is contaminated industrially produced, refrigerated nutritional products, ready to eat without reheating or cooking like soft cheeses, dairy products and smoked fish (Farber and Peterkin 1991).

The murine experimental equivalent of listeriosis represents one of the best understood bacterial infection model to date (Kaufmann 1993;Shen et al. 1998). Concepts such as the inability of antibodies to protect against intracellular pathogens, the importance of activated macrophages in the elimination of intracellular parasites and that T cells are the macrophage activating cells required for cell-mediated immunity, were established based on the murine listeriosis model (Mackaness 1962) (Miki and Mackaness 1964;Mackaness 1969;North 1978).

Infections are usually carried out by intravenous (iv) injections, as mice do not develop a lethal disease after an oral challenge with a reasonable dose. This is due to very poor bacterial translocation across the intestine (Cossart et al. 2003) and poor competitiveness with the commensal gut flora (Manohar et al. 2001).

After experimental iv infection, up to 80% of the inoculum of *L. monocytogenes* is rapidly removed by resident macrophages such as Kupffer cells in the liver. This is followed by an early phase of neutrophil and monocyte influx in the first 24–48 h that form granulomatous lesions at the site of infection (Mackaness 1962;Conlan 1996;Egan and Carding 2000). Resident splenic macrophages remove the remaining 20% of an iv inoculum but fail to inactivate the organisms they ingest. Thus, the spleen appears to be more susceptible than the liver to infection by *L. monocytogenes*. This is in agreement with the fact, that splenectomized mice are more resistant to infection (Skamene and Chayasirisobhon 1977). On the other hand, splenectomized mice fail to develop acquired immunity to reinfection, indicating that the spleen is the place of *Listeria*-specific T cells to be induced by antigen presenting cells (Van den Eertwegh et al. 1992).

1.3.1 Infection cycle of *L. monocytogenes*

The bacterium *L. monocytogenes* has the unusual capacity to enter and to multiply in phagocytes and non-phagocytic cells (Cossart et al. 2003). Various intensively studied tissue culture systems have revealed the infection cycle of this pathogen and the essential key components as well as host cell reactions.

1.3.1.1 Host cell receptors triggered by *L. monocytogenes*

Probably the fate of the bacterium depends on the receptor used for its internalization (Fleming and Campbell 1997). *Listeria* activate a number of different eukaryotic receptors, like the opsonin dependent complement receptors, e.g. C3b and C1q, involved in bacterial uptake by phagocytic cells (Drevets et al. 1993;Alvarez-Dominguez et al. 1993;Croize et al. 1993). Binding to these receptors does not necessarily lead to internalization of bacteria, as macrophages often need to be activated before (Drevets and Campbell 1991;Drevets et al. 1992).

In the absence of serum, bacteria are also efficiently internalized, indicating the role of non-opsonin receptor ligand interactions in *L. monocytogenes* uptake (Pierce et al. 1996). The eukaryotic receptors used by *Listeria* include E-cadherin, Met (Shen et al. 2000), lectins (Facinelli et al. 1998), the macrophage scavenger receptors e.g. macrophage receptor with collagenous structure (Dunne et al. 1994;Greenberg et al. 1996;Pearson 1996), mannose receptors (Fraser et al. 1998) and TLRs (Liu et al. 2002;Gumenscheimer et al. 2002).

1.3.1.2 Invasion and the 'life cycle' of *L. monocytogenes*

Listeria can invade phagocytes and non-phagocytic cells by zipper-like phagocytosis. Entry into non-phagocytic cells is mainly triggered by the two listerial surface proteins, internalin A (InlA) and InlB. These two internalins interact with host cell receptors and mimic the normal cellular ligands (Cossart et al. 2003). InlA interacts only with E-cadherin, while InlB is known to use three different receptors; CR1q, Met or glycosaminoglycans (GAGs) (Cossart et al. 2003). Probably additional bacterial factors are involved, since analysis of the genome of *Listeria* revealed a high number of similar surface proteins (Cossart et al. 2003).

Following entry, bacteria reside within membrane-bound vacuoles for about 30 min, before lysing these structures (Gaillard et al. 1987). After around two hours 50% of bacteria are free in the cytoplasm (Tilney and Portnoy 1989). Once in the cytosol, bacteria replicate, recruit host cell actin filaments, and then move as these filaments organize into long actin tails. When the moving bacteria contact the plasma membrane, they induce the formations of bacteria containing protrusions, which can invaginate into neighboring cells. In the newly infected cells bacteria is surrounded by two plasma membranes, which are then rapidly lysed and a new cycle of multiplication begins (Vazquez-Boland et al. 2001;Cossart et al. 2003).

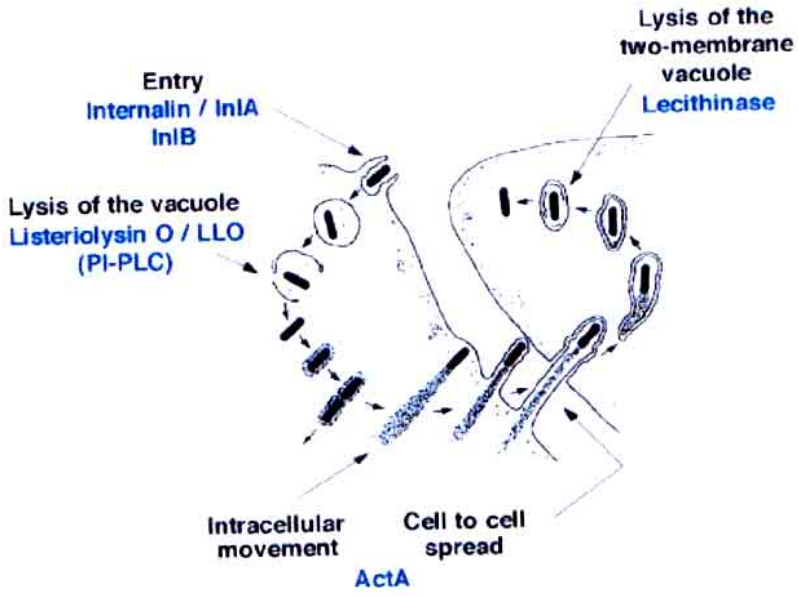

Figure 1.6 Life cycle of *L. monocytogenes.* (Cossart et al. 2003)

1.3.1.3 Virulence factors

To accomplish its life cycle, bacteria use a number of virulence factors. Entry into host cell involves the bacterial surface proteins InlA and InlB, as described above. Little is known about the signaling cascades triggered by *Listeria* in non-phagocytic cells. PI 3-kinase is known to be an essential component of InlB-mediated phagocytosis, since its blockade inhibits this process (Cossart et al. 2003). Products of the reaction catalyzed by PI3K may directly interfere with the actin cytoskeleton in order to induce phagocytosis.

Recently listeriolysin O (LLO), a pore forming toxin and the first virulence factor to be identified in *Listeria* (Gedde et al. 2000), has been accepted as an additional component triggering the entry of *Listeria* into host cell via Ca^{++} mobilization (Cossart 2002;Cossart et al. 2003). With LLO in vacuolar escape synergize phospholipase A (PlcA).

The bacterial surface protein ActA mediates intracellular actin-based movement of *Listeria*. ActA is able to recruit and activate Arp2/3 (actin nucleator protein complex), thereby inducing the formation of actin filaments and generating a crosslinked actin filament network (Welch et al. 1997;Welch et al. 1998;Cossart 2000).

Lysis of the two-membrane vacuole is performed by phosphatidylcholine specific phospholipase (PC-PLC, PlcB, lecithinase) in concert with LLO (Portnoy et al. 2002;Cossart et al. 2003). The PlcB phospholipase is able to degrade most membrane phospholipids. The role of PlcA is not clear, since absence of this phospholipase only lead to a minor defect *in vivo* virulence and in escape from primary vacuoles (Vazquez-Boland et al. 2001).

In addition to the above mentioned virulence factors there are other listerial products, which contribute to survival within the host, although their participation is indirect. For instance the protein p60, a mureine hydrolase, antioxidative factors able to detoxify the endogenous free oxygen radicals, proteins involved in metal ion transport and stress response mediators like ClpC ATPase (Vazquez-Boland et al. 2001).

Six of the key virulence factors are clustered in a single 10 kb chromosomal region (Kuhn et al. 1999), under the absolute control of activator protein PrfA. This cluster is called 'PrfA dependent virulence gene cluster' or '*Listeria* pathogenicity island 1' (LIPI-1) (Figure 1.7a) (Kreft et al. 1999;Chakraborty et al. 2000). The PrfA protein belongs o the Crp-Fnr family of transcriptional activators and recognizes 14 bp sequences called PrfA boxes within target promoters (Sheehan et al. 1996).

Internalins form a separate pathogenicity island, LIPI-2, and are only partially regulated by PrfA (Figure 1.7b)(Dramsi et al. 1993;Engelbrecht et al. 1996).

1.3.2 Pathogenicity

Listeria monocytogenes has the amazing capacity to cross three tight barriers, the intestinal, blood-brain and fetoplacental barriers. The clinical signs of infection are very similar in all susceptible hosts, starting with flu-like symptoms. Although listeriosis is comparatively rare it has a mortality rate reaching 30% in humans, despite of antibiotic treatment (Schuchat et al. 1991;Cossart et al. 2003).

After breaking the intestinal barrier bacteria are engulfed by phagocytes in the lamina propria and the Payer's Patches. They then disseminate via the lymph and blood to the liver and spleen. In immunocompromised hosts, bacteria can further disseminate into the brain and in pregnant women into the placenta. Thus, since *Listeria* can disseminate directly from cell to cell, it circumvents host defenses such as circulating antibodies and complement. This dissemination provides an explanation for the observation that antibodies have little effect, and that immunity to *Listeria* is mainly T-cell mediated.

There are two basic forms of listeriosis: perinatal listeriosis and listeriosis in the adult patient. In both cases clinical forms are either disseminated infection or local infection in the central nervous system (CNS).

Fetomaternal listeriosis results from invasion of the fetus via the placenta and develops as chorioamnionitis. Its consequence is abortion or the birth of a stillborn fetus. Uptake of *Listeria* by the fetus is via the placenta (Abram et al. 2003).

The listerial infection most frequently reported in adults is affecting the CNS (55-70%) and septicemia and bacteremia occurs in 15-50% cases. Other atypical clinical forms are also possible, such as endocarditis.

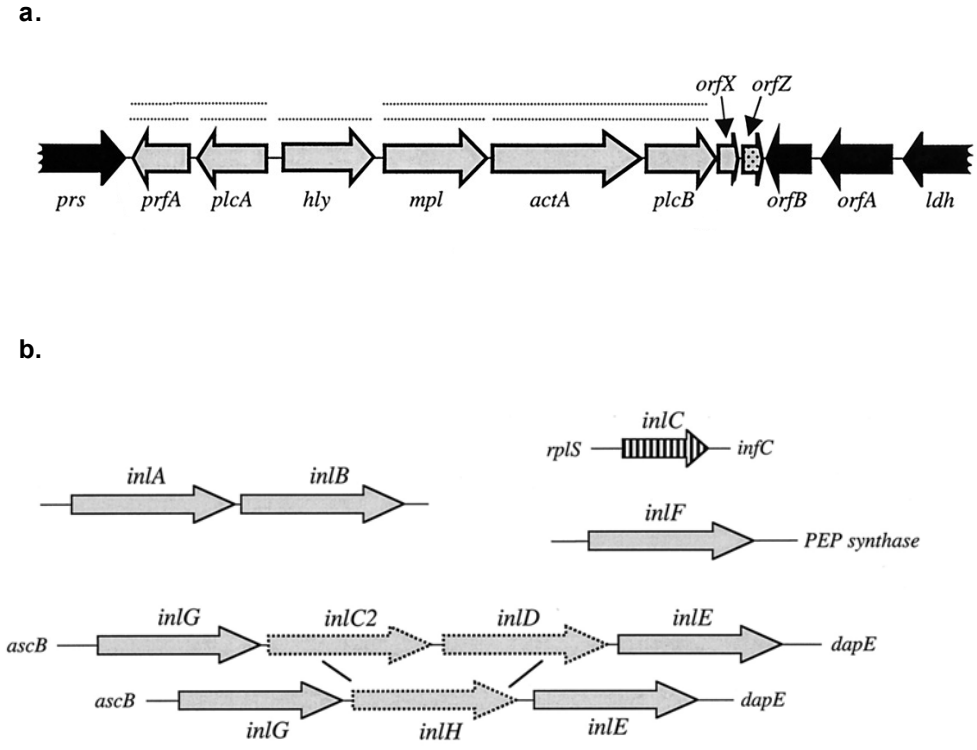

Figure 1.7 Virulence gene cluster. a. Physical and transcriptional organization of the central virulence gene cluster (LIPI-1) of *L. monocytogenes.* Genes belonging to LIPI-1 are in grey, and the flanking loci are in black. Dotted lines above *L. monocytogenes* LIPI-1 genes indicate known transcripts. LIPI-1 is inserted in a chromosomal region delimited by the prs and ldh genes, encoding the housekeeping enzymes phosphoribosyl-pyrophosphate synthase and lactate dehydrogenase, respectively. **b.** Internalin islands and islets of *L. monocytogenes.* The scheme shows the different gene arrangements found for the same internalin locus in two isolates of *L. monocytogenes*, possibly resulting from rearrangements between *inl* genes (*inlH* presumably arose by recombination of the 5'-terminal part of *inlC2* and the 3'-terminal part of *inlD* (Vazquez-Boland et al. 2001).

1.3.3 Innate and adaptive immunity against *Listeria*

The innate response to *Listeria* involves a coordinated interaction between three cell types, macrophages, NK cells and neutrophils (Bancroft et al. 1991;Bancroft 1993;Rogers et al. 1994). Neutrophils play a key role in the early stage of bacterial growth, appearing at the place of infection within 24 hours (Rogers and Unanue 1993; Czuprynski et al. 1994;Conlan and North 1994;Edelson and Unanue 2000) are involved in listerial clearance in the liver.

As described above, experimental infections in mice via the iv route have shown that bacteria are rapidly cleared from the bloodstream by resident macrophages in the spleen and liver. Most of the bacteria accumulate in the Kupffer cells of the liver. Most of the bacteria are destroyed there during the first hours of infection. During these early steps of liver colonization, neutrophils are recruited to the sites of infection, forming micro abscesses (Rogers et al. 1996). Two to four days post infection (PI) neutrophils are gradually replaced by blood derived mononuclear cells together with lymphocytes to form granulomas (Mandel and Cheers 1980;Heymer et al. 1988).

In the spleen, macrophages of the marginal zone are primarily infected. Infected macrophages produce cytokines (IL-12 and TNFα) activating NK cells and stimulating them to produce IFNγ (Unanue 1997). TNFα, in cooperation with IFNγ then leads to the full activation of macrophages via upregulation of MHC II and further cytokine production (Dai et al. 1997a). An alternative source for IFNγ could be splenic $CD8^+$ plasmacytoid DC (Ohteki et al. 1999;Fukao et al. 2000) or, as recently shown, T cells (Bregenholt et al. 2001). This novel observation demonstrates how the adaptive immune response can maintain and influence innate immunity.

Cytokines shown to play a role during the early response to *Listeria* were IL-6, IL-1 together with IL-12, TNFα and IFNγ. (Unanue 1997) and Eta-1 (Ashkar et al. 2000). All these cytokines act through their specific cell membrane receptors, leading to the activation of the NFκB pathway. This results in activation of macrophage, recruitment of other phagocytes as well as attraction of specific T cells. It has been shown in hepatocytes that NFκB activation is essential to clear bacteria from the liver.

At the same time, the anti-inflammatory cytokine IL-10 seems to exert a regulatory function. Absence of IL-10 increased both innate and acquired immune responses in murine listeriosis by elevated macrophage and NK derived pro-inflammatory cytokine responses and by favoring protective Th1 responses. The consequence was an effective control of bacterial

growth and elimination of the invader during primary and secondary infection (Dai et al. 1997b).

Some virulence factors of *L. monocytogenes* are known to trigger particular signaling pathways that lead to regulation of gene expression involved in the host responses. Thus, it has been shown, that bacteria expressing LLO or purified LLO acts as inflammatory stimulus resulting in expression of adhesion molecules, secretion of cytokines and chemokines in macrophages, and in NO release by endothelial cells (Drevets 1997;Kayal et al. 1999;Rose et al. 2001). In this context, LLO has been shown to activate NFκB. Interestingly LLO dependent cell stimulation could be delayed, in comparison to the activation with other inflammatory stimuli, by 30 to 60 minutes PI suggesting the induction of an autocrine loop for NFκB induction, at least in some cell types. In others, NFκB can be induced directly (Kayal et al. 1999;Kayal et al. 2002).

Thus, the transcriptional activator NFκB appears to be the most important upstream element of signaling cascades involved in listerial infection. It activates various genes playing a central role during the inflammatory response, including cytokines (IL-1, IL-6, IL-12, IFNα/β), growth factors, chemokines and adhesion molecules (E-selectin, P-selectin, ICAM-1) (May and Ghosh 1998). LLO has also been shown to activate signaling pathways other than NFκB, such as the MAP-kinase pathway (Tang et al. 1996) and phosphatidyl inositol-induced lipid mediator generation (Sibelius et al. 1996). In addition, LLO can induce apoptosis in murine DCs (Guzman et al. 1996) and hepatocytes (Rogers et al. 1996).

Other virulence factors or listerial components induce signals via kinases like PKC, the MAP kinases like p38, JNK and ERK-1, 2. This seems to play a role in *Listeria* uptake and invasion for example by regulation of cytoskeletal changes (Guzman et al. 1996;Tang et al. 1998;Kuhn and Goebel 2000).

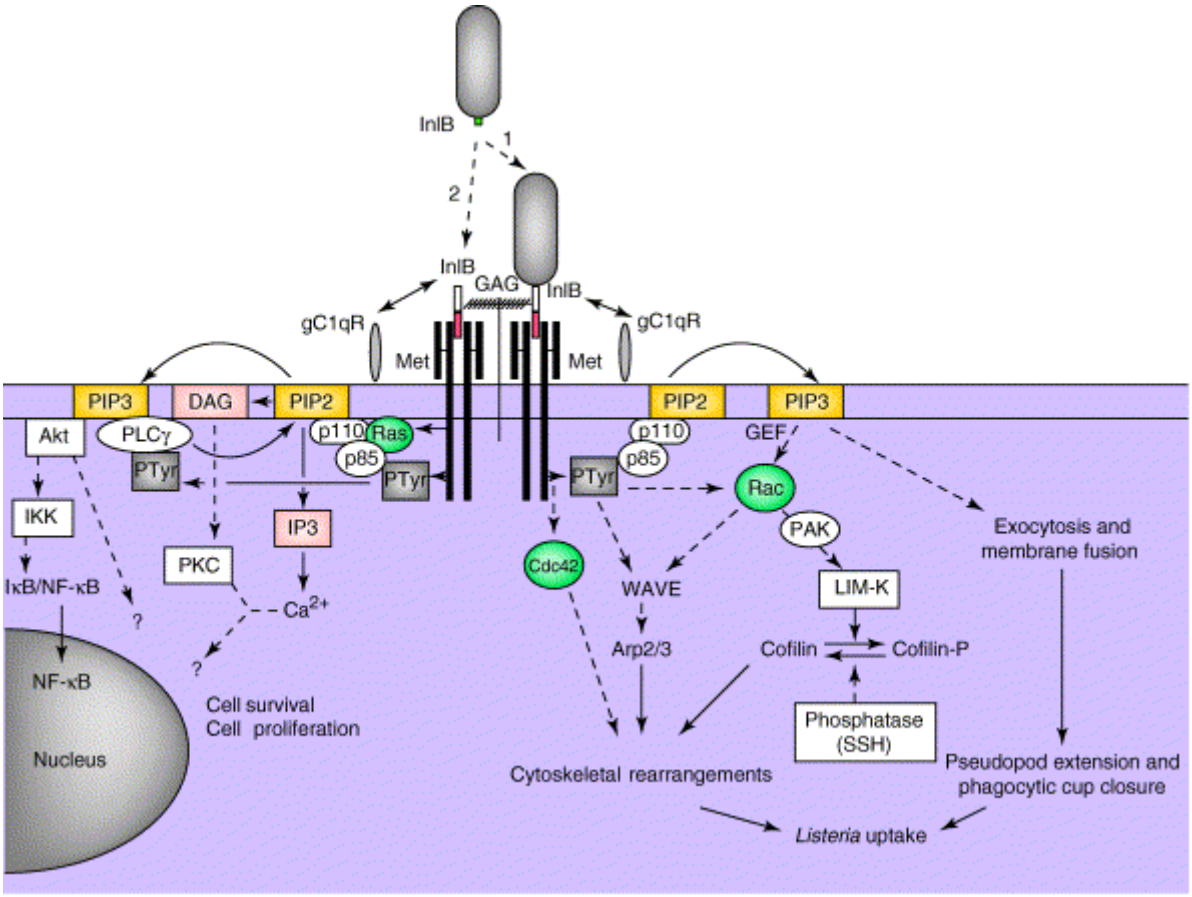

Figure 1.8 Signaling pathways mediated by InlB. Normally InlB is buried in the bacterial cell wall, where it is protected from proteolytic degradation and external aggressive agents. The surface-bound InlB protein triggers entry of the bacterium. The interaction between InlB and Met induces the recruitment of adaptor proteins, some of which become tyrosine-phosphorylated, and activated. Downstream events induce both rearrangements of the actin cytoskeleton and membrane reorganization, leading to uptake of the bacterium. Other downstream events, involving the activation of other enzymes such as phospholipase C- 1 and the nuclear factor NF-κB might affect the fate of the bacterium in the cell and/or the behavior of the host cell. Dashed arrows indicate hypothetical steps (Cossart et al. 2003).

Thus signals via LLO together with lipases, LTA and internalins (Figure 1.8 and 1.9) result in activating the innate immune system that is sufficient to control a sublethal infection of mice with *L. monocytogenes*, the adaptive immune system seems not to be required. However, without an adaptive immune response listerial infection becomes chronic (Mombaerts et al. 1993). Especially γδ T cells, appearing as soon as 1-3 days PI (Ohga et al. 1990;Hiromatsu et al. 1992), seems to be required in the resolution of the cellular response to *Listeria* and prevention of chronic inflammation. Mice depleted in these cells (TCR- $\gamma\delta^{-/-}$) and infected with *L. monocytogenes*, exhibited exaggerated tissue inflammation and necrosis after infection

(Fu et al. 1994). Since it is well known that γδT cells are not involved in bacterial clearance or in the generation of T cell memory, it was postulated that these cells have an unique ability to prevent the development of chronic inflammation after infection, by inducing cell death in activated macrophages and, in the process, promoting the resolution of inflammation after the termination of the immune response to *Listeria* (Egan and Carding 2000;Dalton et al. 2003).

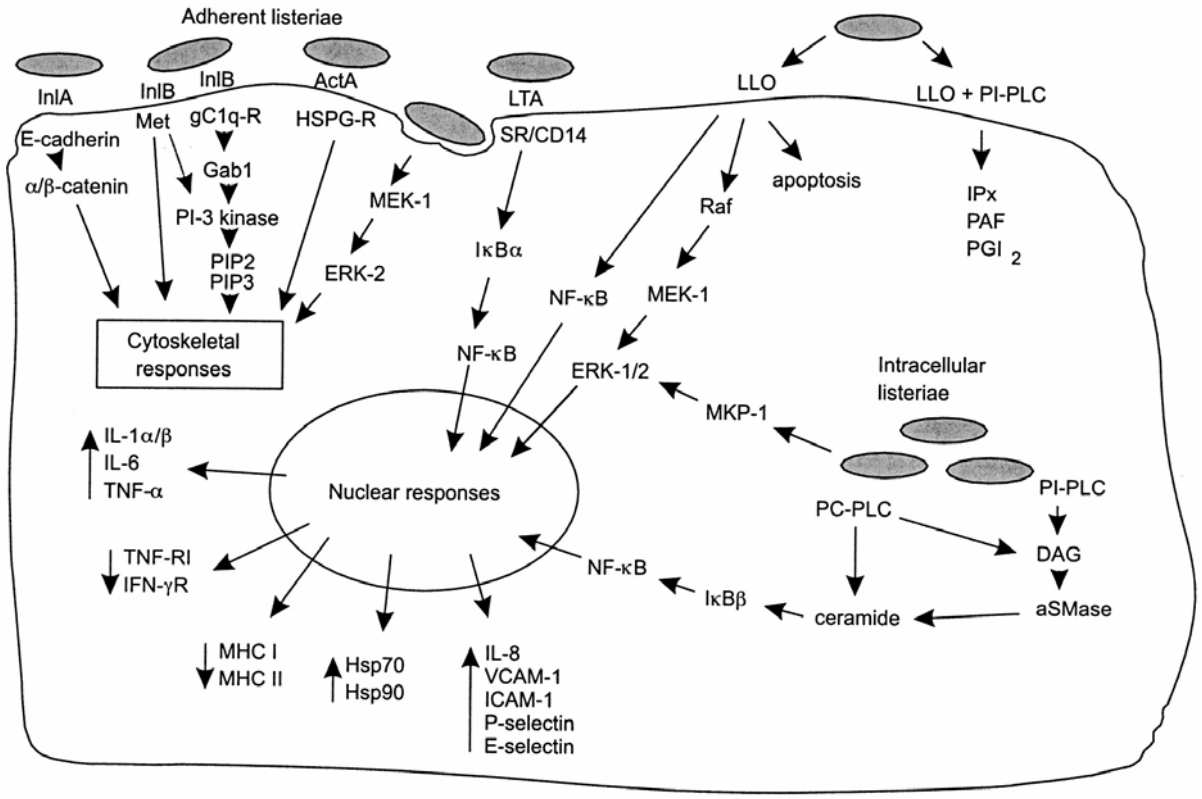

Figure 1.9 Signal transduction pathways and host cell responses. Summary of modulated signal transduction pathways and host cell responses identified during *L. monocytogenes* infection of murine macrophages. Abbreviations: αSMase, acidic sphingomyelinase; gC1q-R, complement C1q receptor; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; HSPG-R, HSPG receptor; ICAM-1, intercellular adhesion molecule 1; IFN-γ R, IFNγ receptor; IPx, inositolphosphates; LTA, lipoteichoic acid; MEK-1, mitogen-activated protein kinase kinase 1; Met, receptor tyrosine kinase for HGF; MKP-1, mitogen-activated protein kinase phosphatase 1; PAF, platelet-activating factor; PGI_2, prostaglandin I_2; PIP2, phosphatidylinositol-(3,4)-bisphosphate; PIP3, phosphatidylinositol-(3,4,5)-trisphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; SR, scavenger receptor; TNF-RI, TNF receptor type 1; VCAM-1, vascular cell adhesion molecule-1 (Kuhn and Goebel 1998).

In parallel one can observe an early influx of regulatory NK T cells bearing the NK1.1 marker into the sites of infection. Since these cells are able to produce either IL-4 (Bendelac et al. 1996) or IFNγ (Ogasawara et al. 1998) they are considered as regulatory cells directing an anti-*Listeria* immune response toward either the Th1 or Th2 pathway (Skeen et al. 2001).

Consistent with a response dominated by Th1 cells, is the rapid loss of IL-4 producing NK T cells in liver during early infection (Kaufmann and Kaplan 1996)

During the early phase of *Listeria* infection most $CD8^+$ T cells in the spleen, regardless of their specificity, become activated. This early activation, probably due to type I IFNs is transient and at around day 2 all such unspecific cells start to die via apoptosis (Jiang et al. 2003). A specific immune response is initiated by day 3-4, and Ag-specific $CD8^+$ and $CD4^+$ T cells migrate into the spleen (Jiang et al. 2003). Generation of specific $CD8^+$ T cells appeared essential for the complete clearance of *L. monocytogenes* and for subsequent memory responses (Bregenholt et al. 2001). $CD4^+$ T cells seems to be involved in the delayed-type hypersensivity and granuloma formation (Mielke et al. 1988;Harty et al. 1992). Again the influx of these cells is dependent on the presence of particular virulence factors, as mutants that lack ActA and PlcA, do not support the immigration of granuloma forming $CD4^+$ T cells.

Innate and adaptive immunity against *L. monocytogenes* is schematically depicted in Figure 1.10

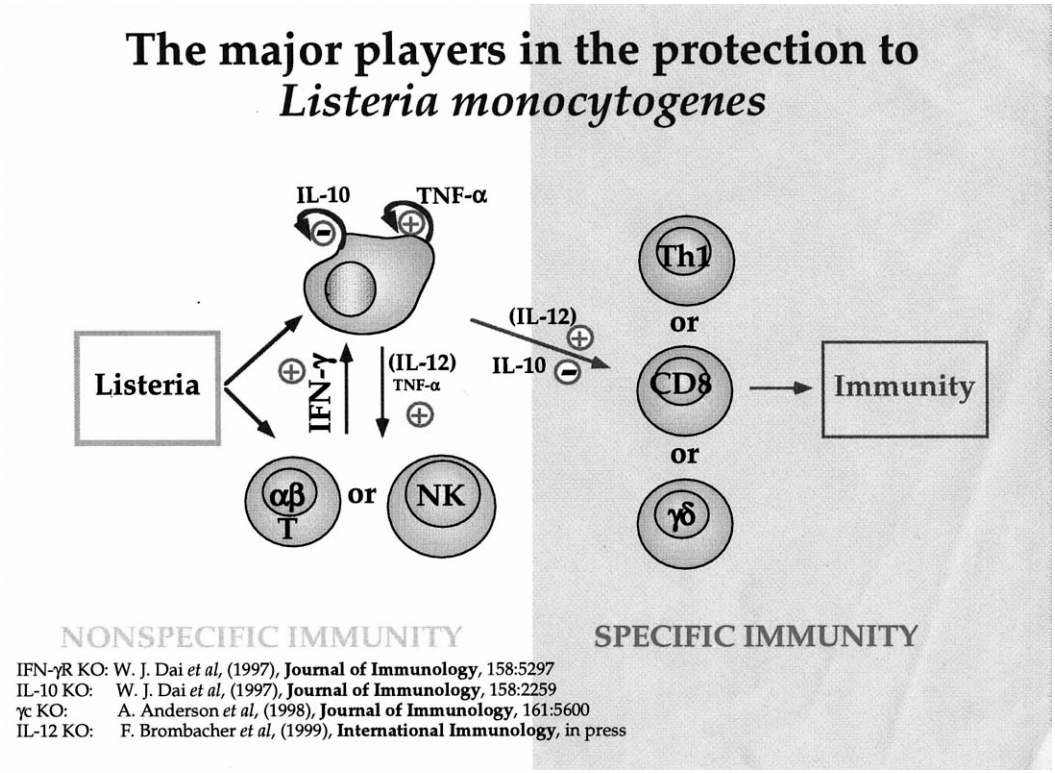

Figure 1.10 Innate and adaptive anti-*Listeria* response. (Bregenholt et al. 2001)

2 Materials and methods

2.1 Bacterial strains

L. monocytogenes EGDe:	wild type strain serotype 1/2a (Leinmaister-Wächter und Chakraborty, 1989)
L. monocytogenes Δhly2:	isogenic hly in frame deletion mutant of EGDe lacks LLO expression, non-hemolytic (Guzman et al., 1995)

2.2 Cell lines

J774, Raw 264.7 monocyte-macrophage cell lines generated from BALB/c mice

2.3 Mice

Female BALB/c (H-2^d), DBA/2 (H-2^d) and C57Bl/6 (H-2^b) mice were purchased from Harlan Winkelman (Borchen, Germany) and used for all experiments at an age of 8-12 weeks.

2.4 Culture media

2.4.1 BHI broth for bacteria

Brain Heart Infusion (BHI) broth or agar plates (Difco, Detroit, USA) were used for the culture of *L. monocytogenes*.

2.4.2 IMDM complete medium for cells

Iscove's modified Dulbecco's modified Eagle's medium (IMDM-medium, Gibco BRL, Eggstein, Germany) was used for cell culture. IMDM was prepared according to manufacturer's protocol and then enriched with 10% FCS (Integro, Zandam, The Nederland), 2mM L-glutamine, 0.25 μM β-mercaptoethanol (Serva, Heidelberg, Germany).

For the culture of cells infected with *L. monocytogenes* IMDM with 20 μg/ml of gentamycin was used to avoid reinfection (Cytogen, Ober-Mörlen, Germany).

2.5 Culture of bacteria

Bacteria were grown in BHI (brain heart infusion) broth at 37°C overnight, then suspensions were diluted 1/5 in fresh medium and incubated for the additional 3 h at 37°C, until they reached mid-log phase. Depending on the experiment, bacteria were then washed and resuspended in either sterile PBS (infection of mice) or tissue culture medium IMDM (*in vitro* infection of macrophage).

2.5.1 Estimation of numbers of bacteria

To determine bacteria concentration in the sample, bacteria were washed, diluted in sterile PBS and then OD was measured at 600nm. The value at OD_{600} was compared with an established growth curve of *L. monocytogenes* (Table 2.1 Hense, PhD thesis, TU Braunschweig, 1999)

OD_{600}	CFU/ml
0.05	4.2×10^6
0.07	8.56×10^6
0.016	1.34×10^7
0.024	2.17×10^7
0.042	5.53×10^7
0.067	1.1×10^8
0.099	1.68×10^8
0.181	5.44×10^8
0.427	1.53×10^9
0.583	4.57×10^9
0.706	1.57×10^{10}
0.775	3.3×10^{10}
0.841	5.64×10^{10}
0.862	1.52×10^{11}

Table 2.1 Growth curve of *L. monocytogenes*

2.5.2 Evaluation of the number of intracellular bacteria

Lysates of infected cells were prepared by washing cells with PBS and then adding 500µl of 0.1% Triton X-100 in H_2O per well. Cells then were incubated for 30 min at room

temperature (RT). To determine the number of the viable intracellular bacteria, serial dilutions of the lysates were plated on the BHI agar plates.

2.6 Isolation and cultivation of macrophages

2.6.1 Isolation of the cells

Bone marrow derived macrophages (BMDM) were isolated from the bone marrow of hind legs of mice, cell were allowed to adhere and settle in 100 mm Petri dishes (Sarsted, Nümbrecht, Germany) at the final density $5x10^6$ in 15 ml of IMDM medium supplemented with 200 U/ml rmGM-CSF. Cells were grown for 10 days. On day 3, 5 and 7 culture medium containing loosely and non-adherent cells was removed and new medium with GM-CSF was added. For infection, after 10 days macrophages were harvested using a rubber policeman stained and transferred into a 25 cm^2 flasks at a density $4x10^5$/ml and incubated overnight.

Purity of BMDMs was analyzed using the flow cytometer staining of the surface markers: CD11b (Mac-1), F4/80, CD11c (N418), CD45 (B220), ICAM-1, CD40, Kl-II and Gr-1. The purity of macrophages was above 80 % (Figure 2.1).

After overnight incubation, cells were washed with PBS and used for infection.

2.6.2 Determination of the macrophage number

BMDM were harvested using a rubber policeman, resuspended in PBS, diluted with Trypan-blue 1:1 and counted in the Neubauer Zählkamer 0.1mm/0.025mm^2 (Brand, Wertheim, Germany).

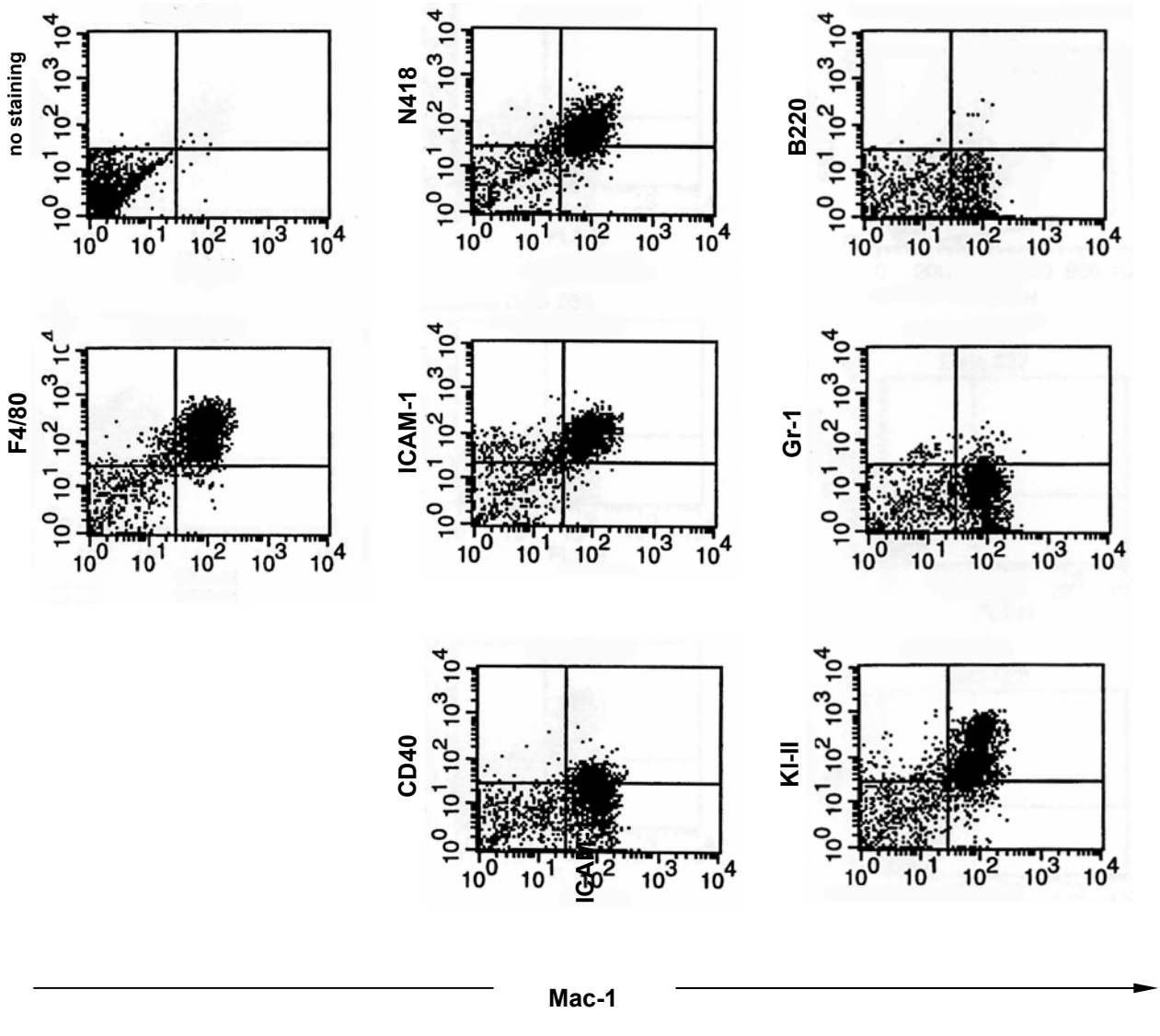

Figure 2.1 Bone marrow macrophages isolated from BALB/c female mice. Macrophages were isolated from the bone marrow of mice hind legs and settled in the 100 mm Petri dish (Sarsted, Nümbrecht, Germany) at the final density $5x10^6$ in 15 ml of IMDM medium supplemented with 200 U/ml rmGM-CSF. Cells were grown for 10 days, at day 3, 5 and 7 culture medium containing loosely and non-adherent cells was removed and the new one with GM-CSF was added. Analysis of the cells was made using FACSCalibur and the software CellQuestPro.

2.7 *In vitro* infection of macrophages with *L. monocytogenes*

2.7.1 Determination of optimal infection conditions

For analysis of regulated genes in infected macrophages optimal assay conditions needed to be determined. As many cells as possible should be infected and detrimental effects of virulence factors like listeriolysin should be at a minimum. It is also important to mention that cells survive a listerial infection only for a certain time. Therefore macrophages were infected at different multiplicities of infection (MOI) and production of IL-6 was measured by ELISA as indicator for induction of gene expression. At the same time CFU of intracellular bacteria and viability of the macrophages were estimated. Cells started to die around 8 h after infection even at low MOI. Thus, in the following experiments macrophages were infected at a MOI that induced strong IL-6 production and samples were taken at 2 and 6 h PI (post infection) in order to avoid problems for RNA preparation due to dying or dead cells.

2.7.2 Infection

Macrophages were plated at $4x10^4$cells/ml in 5ml in standard IMDM without antibiotics in a 25 cm^2 flask. After overnight incubation cells were infected with *L. monocytogenes* with the optimal multiplicity of infection (MOI), established before - J774 (MOI 10), BMDM (50), Raw264.7(25). After one hour, gentamycin was added to kill extracellular bacteria. After 2 and 6 h cells were harvested for RNA extraction using a rubber policeman.

2.8 Infection of mice with *L. monocytogenes*

2.8.1 Intravenous infection

Female mice were infected intravenously with $2x10^3$ (low dose infection) or $5x10^5$ (a high dose infection) CFU of *L. monocytogenes.* Number of injected *Listeria* was confirmed by plating an aliquot of the inoculum on BHI plates. After 0, 1, 2, 3 days or 0, 2, 4, 6, 24 h respectively, spleens were removed, part of the organs used for RNA extraction and aliquots

were frozen in liquid nitrogen for histology, while the rest of the organ homogenized in PBS supplemented with 0.2% NP-40 and plated.

2.8.2 Isolation of adherent cells from the spleens of infected mice

Spleen cells from infected animals were resuspended for two min in erythrocyte lysis buffer (ACK buffer), and then single cell suspensions were washed three times in IMDM supplemented with antibiotics (penicillin 100µg/ml, streptavidin 100µg/ml, gentamycin 20 µg/ml). Cell suspensions were incubated for 1 h at 37^{o}C in a humidified 10% CO_2 atmosphere on Petri plates, after this time, plates were washed with PBS and adherent cells removed from the plate using rubber policeman.

ACK buffer: 0,15M NH_4Cl, 10mM $KHCO_3$, 0,1mM Na_2EDTA – pH 7,2-7,4

2.8.3 Collagenase D digestion of the spleen

To obtain plasmacytoid DCs from the spleen, collagenase treatment was employed. Spleens were fragmented and incubated with 2 ml of the collagenase D (0.5mg/ml) in PBS for 30 min at 37^{o}C in a humidified 10% CO_2 atmosphere. Subsequently digested tissues were passed through a mesh, washed, and 400µl of 0.1 M EDTA was added. After 5 min incubation in RT cells were washed and resuspended in ACK buffer for two min in room temperature. After subsequent washing cells were stained as described below.

2.8.4 Evaluation of the number of *L. monocytogenes* in the spleen

Mice were sacrificed at indicated time points, spleens were removed, fragments of them were homogenized in 1 ml of PBS supplemented with 0.2% NP-40. Serial dilutions of homogenates were plated on BHI agar plates in duplicates and CFU (Colony Forming Units) were estimated after overnight incubation at 37°C.

2.9 Preparation of RNA and DNA

2.9.1 RNA extraction

Mice were killed by CO_2 treatment or cervical dislocation, spleens removed and spleen cells isolated as described above. For array hybridization, total cellular RNA was extracted from cells using TRIZOL Reagent (Gibco BRL, Eggstein, Germany), according to the manufacturer's instructions. For RT-PCR and Real-Time RT-PCR total cellular RNA was isolated using RNeasy kit (QIAGEN, Hilden, Germany), according to the provided protocol.

2.9.2 DNase treatment

To eliminate possible residual DNA contamination in RNA preparations DNA-free Kit (Ambion, Wiesbaden, Germany) was used according to the supplied protocol. Extracted RNA was incubated with DNase (2 units) at 37^{o}C, after 30 min treated with DNase Inactivator, centrifuged at 10.000x g for 1 min and supernatants collected.

2.9.3 Determination of RNA concentration

For optical estimation of RNA concentration in the samples the extinction was measured at 260 nm. The photometer used was the BioPhotometer (Eppendorf, Hamburg, Germany). For the subsequent cDNA preparation 1µg of RNA was used. 1 OD ≈ 30µg RNA.

2.10 Micro- and Macro Arrays

An approach to analyze multiple genes simultaneously is the hybridization of the entire cDNA population to nucleic acid arrays. This is a method adopted for high-throughput analysis of gene expression. The technology has a wide range of applications, including investigating normal biological and disease processes, profiling differential gene expression, and discovering therapeutic and diagnostic drug targets.

2.10.1 Macroarrays

CLONTECH's Atlas cDNA Expression Arrays (BD Clontech, Heidelberg, Germany) include hundreds of cDNA spotted on positively charged nylon membranes. Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several housekeeping genes as positive controls, to allow the normalization of mRNA abundance. Atlas Arrays are carefully designed to include genes of great interest to researchers in many fields. The cDNA immobilized on each array have been specially prepared to minimize nonspecific hybridization – each fragment is 200 to 600 bp (base pairs) and has been amplified from a region of mRNA that lacks the poly-A tail, repetitive elements or other highly homologous sequences.

Detection of rare transcripts depends on the quality of RNA samples; successful synthesis and labeling of a cDNA probe with high specific activity, and requires low level of nonspecific hybridization.

In the presented experiment, RNA was extracted from $1x10^7$ infected cells using Tri-Reagent LS (Molecular Research Center, Steinbach, Germany) according to manufacturer's instructions. Total RNA from cells was used as a template for synthesis of ^{32}P-labeled cDNA probes. Probes were then hybridized to the gene array membranes containing various mouse cDNAs (Atlas mouse 1 and 2 Array, BD Clontech, Heidelberg, Germany). After hybridization and washing, membranes were exposed to Phosphor Imaging Screens (Fuji, Düsseldorf, Germany) and developed using a Fuji Bas 2500 16-bit image analysis system. Analysis was carried out in duplicates and evaluated with Array-Vision software 5.1 (Imaging Research, Mering, Germany). Expression of a total of 1176 genes was analyzed this way.

2.10.2 Microarrays

The comprehensive study of gene expression in cells has recently been made possible by the development of gene microarrays like Affymetrix high-density oligonucleotide array. These arrays allow for highly parallel, reproducible, quantification of gene expression levels.

RNA was isolated from $1x10^7$ infected cells and double stranded cDNA was prepared. An *in vitro* transcription reaction was performed to produce biotin-labeled cRNA. Subsequently, the hybridization cocktail was prepared (fragmented cRNA, probe array controls, BSA and herring sperm DNA) then hybridized to the oligonucleotide spots on the micro array (Mu 11K

– 11,000 murine genes and EST clusters, Affymetrix, High Wycombe, UK) for 16 h at 45°C. Following washing, staining of the hybridized probe array was performed using streptavidine (SA) conjugated with phycoerythrin. The whole reaction was done in automated fluidics station according to the manufacturer's protocol. Data were then scanned and the resultant image analyzed by GeneChip software provided by the vender – Affymetrix GeneChip (High Wycombe, UK). The scanned images contain quantitative information about fluorescence bound to probes of genes. The levels of expression of transcripts were calculated and expressed as average differences. Data from two independent infections were compared. Expression levels differing more than two-fold were considered significant.

2.11 Preparation of cDNA

To prepare cDNA, 1µg of RNA was used. RNA was first incubated with 1µM of oligo dT, for 10 min at 70°C. After cooling on ice, reaction mixture (Invitrogen, Karlsruhe, Germany) was added and reverse transcription conducted.

Reaction mixture:	First Strand Buffer
	0.1 M DTT
	dNTPs (200µM)
	RNasin (40 U/µl)
	Superscript II (200 U/µl)
RT program:	42°C for 1 h, 95°C for 2 min
dNTPs:	100 mM stock solution of dATP, dTTP, dGTP and dCTP (Amersham Pharmacia Biotech, Freiburg, Germany) diluted in H_2O

2.12 Polymerase Chain Reaction (PCR)

To determine levels of several cytokines and chemokines, spleens were isolated from *Listeria* infected mice, mRNA was isolated, cDNA was transcribed from 1µg of RNA (as described above) and 1µl employed in either RT-PCR or Real-Time RT-PCR.

2.12.1 Oligonucleotides

Primers for RT-PCR; semi-nested PCR and Real-Time RT-PCR were selected by DNASTAR, Primer Select software (Lasergene, Madison, USA) and purchased from Promega (Promega, Madison, USA). Primers selected for PCR should possibly include intron sequences, what allows controlling genomic DNA contamination, should not contain repetitive sequences and their length should be around 20 bp. For Real-Time RT-PCR the length of product is very important – it should not extent 200 bp.

Primer name: (forward; reverse)

CCL2: (CTGTGCTGACCCCAAGAAGG; AATTAAGGCATCACAGTCCGAGTC),

LTRIAL1: (CAGCACCAGCACCAGCCAACT),

CCL3: (CTGCCCTTGCTGTTCTTCTCTGTA; GATCTGCCGGTTTCTCTTAGTCA),

CCL4: (ACCATGAAGCTCTGCGTGTCTG; GTGAAGCTGCCGGG AGGTGTA),

CCL5: (GCTCCAATCTTGCAGTCGTGTTT; GACCGGAGTGGGAGTAGGGGATTA),

CCL7: (ACGCTTCTGTGCCTGCTGCTC; CCTCCTCGACCCACTTCTGATG),

CCL12: (TCCACACTTCT ATGCCTCCTGCTC; ACTGGCTGCTTGTGATTCTCCTGT),

CXCL2: (TGAGTGTGACGCCCCCAGGAC; TCAGACAGCGAGGCACATCAGGTA),

CXCL10: (GATGACGGGCCAGTGAGAATGAG; CTGGGTAAAGGGGAGTGATGGAGA),

IL-1β: (TTGACGGACCCCAAAAGATG; AGAAGGTGCTCATGTCCTCA),

IL-6: (GTTCTCTGGGAAATCGTGGA; TGTACTCCAGGTAGCTATGG),

IL-10: (CTGGACAACATACTGCTAACCGACTC; ATTTCTGGGCCATGCTTCTCTGC),

IL-12 p40: (CAGTACACCTGCCACAAAGGA; GTGTGACCTTCTCTGCAGACA),

TNFα: (TCTCATCAGTTCTATGGCCC; GGGAGTAGACAAGGTACAAC),

IFNα4: (GTGCTTTCCTCATGATCC; GGTTGAGGAAGAGAGGG),

IFNnonα4: (ARSYTGTSTGATGCARCAGGT; GGWACACAGTGATCCTGTGG),

IFNβ: (ACCACAGCCCTCTCCATCAACTA; CTCTTCTGCATCTTCTCCGTCATC),

IFNγ: (GCTCTGAGACAATGAACGCT; AAAGAGATAATCTGGCTCTGC),

iNOS: (CACACCAGGACCCTCCTCGA; CGGTTACTCCATGAGTCGCA)

RPS9: (CTGGACGAGGGCAAGATGAAGC; TGACGTTGGCGGATGAGCACA)

2.12.2 RT-PCR

RT-PCR was conducted using the thermocycler PCRExpress (Hybaid, MWG, Ebersberg, Germany). Amount of cDNA and efficiency of reaction were controlled using primers specific for the housekeeping gene – RPS9.

Reaction mix:	10 x PCR Buffer w/$MgCl_2$ (Promega, Madison, USA)
	200 µM nucleotide mix (dNTPs)
	10 pmol primermix (forward and reverse primer 1:1)
	1U Taq Polymerase (Promega, Madison, USA)
PCR program:	1 min 94^{o}C, 30 cycles of 94^{o}C for 20 sec, 58^{o}C for 20 sec and 72^{o}C for 20 sec. The final extension at 72^{o}C for 5 min.
Reaction tubes:	0.2 ml (Applied Biosystems, Hamburg, Germany)

2.12.3 Semi-nested PCR

Semi-nested PCR is designed to detect mRNA of extremely abundance. After reverse transcription, the cDNA is amplified in the conventional way. Subsequently a second PCR is performed by adding to the first amplification product a new primer pair comprised of one used in first reaction and second flanking shorter fragment of desired product.

Semi-nested PCR was performed to visualize CCL2 chemokine, at the early stage of low dose *Listeria* infection. The expression of this chemokine is then too low to see the product on the agarose gel after standard RT-PCR reaction. Primers CCL2; forward and reverse were used for the first reaction, then tube was cooled on ice, another primer pair comprised of forward LTRIAL1 and reverse CCL2 was added to reaction, followed by the next run of RT-PCR.

RT-PCR protocols:	First and second amplification as previously described

2.12.4 Real-Time RT-PCR

Real-Time RT-PCR is designed to quantitate the cDNA amount. Amplification of the cDNA is currently monitored during the course of PCR by measuring fluorescence of the sample. Fluorescence is due to the double-strand DNA-specific dye SYBR Green (Applied Biosystem, Hamburg, Germany), which binds double-stranded DNA, and upon excitation emits light. For every pair of primers, during reaction the standard curve is prepared and then obtained results are compared to it. Additionally this method allows following amplification curve what prevents measuring the product amounts in the plateau of reaction.

Real-Time RT-PCR was conducted using a SYBR Green PCR Master Mix kit (Applied Biosystem, Hamburg, Germany) in the GeneAmp 5700 Sequence Detection System (Applied Biosystem, Hamburg, Germany). All reactions were conducted in duplicates. Results were normalized using the housekeeping gene RPS9.

Reaction tubes: 0.2 ml (Applied Biosystems, Hamburg, Germany)

2.13 Agarose gel electrophoresis

To separate linearized, double-stranded DNA fragments the agarose gel electrophoresis was applied. The migration of DNA in an electric field depends on the molecular size of the fragments. The size then can be estimated using DNA marker of known size. In this study gels were used at 2% concentration of agarose (Appligene, Heidelberg, Germany) in TBE buffer and run at 100-110 V. DNA fragments were visualized using UV light, after ethidium bromide staining, and then documented.

TBE buffer 90mM Tris-Borat, 2mM EDTA, 0.11%(v/v) acetic acid

Marker DNA: Smart DNA ladder 100 bp (MBI-Fermentas, St Leon-Rot, Germany)

Electrophoresis was conducted using the Horizon 11.14 system (Gibco BRL, Eggenstein, Germany)

2.14 Immunological methods

2.14.1 Antibodies

rabbit-anti-*L. monocytogenes* (Dunn Labortechnik, Asbach, Germany),

rat-anti-mouse ERTR-9 biotin (BMA Biomedicals, Augst, Switzerland),

rat-anti-mouse MOMA-1 biotin (BMA Biomedicals, Augst, Switzerland),

rat-anti-mouse MOMA-1 PE (Serotec, Düsseldorf, Germany),

rat-anti-mouse DX5 (Pharmingen, San Diego, USA),

rat-anti-mouse Gr-1 biotinylated (Pharmingen, San Diego, USA),

rat-anti-mouse B220 FITC (Pharmingen, San Diego, USA),

rat-anti-mouse B220 APC (Pharmingen, San Diego, USA),

hamster-anti-murine CD3 (Pharmingen, San Diego, USA),

goat-anti-mouse JE/CCL2 Affinity Purified Polyclonal Ab (R&D Systems, Wiesbaden, Germany),

goat-anti-rabbit AMCA (Sigma-Aldrich, Deisenhofen, Germany),

goat-anti rabbit FITC (Sigma-Aldrich, Deisenhofen, Germany)

goat-anti-rabbit PO (Dianova, Hamburg, Germany)

rat-anti-mouse Fc receptor (Pharmingen, San Diego, USA),

* rat-anti-mouse Mac-1 biotin (anti-Mac-1, clone: M1/70.15.11.5, ATCC),

* rat-anti-mouse CD11c biotin (N418, clone: CHB 229, ATCC),

* rat-anti-mouse F4/80 biotin (F4/80, clone: HB 198, ATCC)

* The three latter antibodies were isolated from culture supernatants and biotinylated according to standard procedures.

2.14.2 Flow cytometry

Flow cytometry is able to determine size and granularity of cells. When stained with appropriate antibodies conjugated with fluorescent dyes this method allows the sensitive detection of cell surface molecules.

For the analysis by flow cytometry, cells were obtained by flushing spleens with IMDM enriched with gentamycin (20 µg/ml) and erythrocytes lysis. Cells were then stained in 96 well plates (CellStar, Greiner Labortechnik, Frickenhausen, Germany). Single cell suspensions were prepared in FACS buffer at a density $5x10^5$ cell per well. Cells were first treated with anti-mouse FcR Abs for 15 min on ice, to block Fc receptors, then stained for 15 min on ice, using antibodies (Abs) described above and washed. Biotinylated antibodies were revealed using streptavidin conjugated with APC (SA-APC) or SA-PE (Pharmingen, San Diego, CA), for 10 min on ice. After subsequent washing and staining for the dead cells with 1 µg/ml of propidium iodide (Sigma-Aldrich, Deisenhofen, Germany) flow cytometry was performed using FACSCalibur (Becton Dickinson, Heidelberg, Germany). Data were then analyzed with CellQuestPro software (Becton Dickinson, Heidelberg, Germany).

FACS buffer: 2% FCS, 2mM EDTA and 0,1% Azid in PBS

2.14.3 Flow cytometric cell sorting

The ability of flow cytometers to evaluate cells at an extremely rapid rate (e.g. up to 20,000 events per second) makes this technology ideally suited for the reliable and accurate quantitative analysis of selected physical properties of cells of interest by deflecting the droplets containing single cells for collection and the use of multiple fluorochromes (e.g. up to eight distinct fluorescent probes reacting with different cell associated molecules) simultaneously, cell sorting by flow cytometry can separate complex mixtures of cells on the basis of multiple marker expression. The sensitivity of these instruments allows the detection of as few as 50 molecules per cell.

For cell sorting in this work a FACS Vantage DiVa was used**.** The Vantage had been upgraded with the Digital option (DiVa) and high speed multi-laser sorts of up to four populations simultaneously can now be performed with throughputs near 16,000 cells/second.

To sort distinct population of spleen cells, single cell suspensions were obtained as described for flow cytometry. Cells were then depleted in $CD19^+$ and $CD8^+$ cells using MACS (see

2.14.4) and stained with the specific Abs in FACS buffer (FACS staining, as described above). For each sorting experiment pools of cells from 10 mice were used. Sorted cells were reanalyzed and the purity was 90-95%.

2.14.4 Magnetic cell sorting

Magnetic cell sorting (MACS), developed to separate cells according to the surface markers labeled by MACS colloidal super-paramagnetic MicroBeads conjugated to antibody (Miltenyi Biotec, Bergisch Gladbach, Germany).

To deplete spleen cell population for $CD19^+$ and $CD8^+$ cells, cell suspensions were prepared, as described for flow cytometry. Cells in MACS buffer were then magnetically labeled with MicroBeads conjugated with rat-anti-CD19 and rat-anti-CD8 at 4^oC for 30 min. After subsequent washing, cells were diluted in MACS buffer, loaded on a MACS column and placed in the magnetic field of an autoMACS^{TM} Separator (AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The magnetically labeled cells were retained in the column, while the unlabeled run through. Obtained cells were then used for a further analysis and sorting. The beads are small enough to allow analysis in flow cytometry without the need of stripping the cells off the beads.

rat-anti-mouse CD19 beads (Miltenyi Biotec, Bergisch Gladbach, Germany)

rat-anti-mouse CD8 beads (Miltenyi Biotec, Bergisch Gladbach, Germany)

MACS buffer: 2mM EDTA in PBS, pH 7.2

2.14.5 ELISA

The ELISA (Enzyme Linked Immunosorbent Assay) can be employed for quantitative estimating ng/ml to pg/ml of secreted antigens e.g. cytokines in solution.

Sandwich ELISA was performed, by absorbing the purified Abs non-covalently onto the plastic microwell plates. These antibodies can capture soluble antigen from the sample, which can then be detected using a secondary antibody that is specific for a different epitope on the same antigen and is conjugated to the enzyme – horseradish peroxidase. This enzyme subsequently catalytically converts a chromogenic substrate, leading to the color reaction,

which can be detected spectrophotometrically using an ELISA reader at an appropriate wavelength.

Plates were coated overnight with antibodies diluted in Coating-Buffer at 4°C. After washing, remaining binding sites were then blocked using 5% FCS in PBS for 1 h at 37°C. After washing, 100µl of the supernatants were added to the plate for a 3 h at 37°C. Subsequently, biotinylated secondary Abs were used (1h, 37°C), followed by PO conjugated streptavidine: SA-PO (Sigma-Aldrich, Deisenhofen, Germany). Color reaction was obtained by adding OPD – the substrate for peroxidase. Results were read by ELISA-Reader at the wavelength 490 nm.

Coating Buffer: 0,1 M Na_2CO_3, 0,1 M $NaHCO_3$; pH 9,6 in H_2O

Washing Buffer: 0,1% Tween 20 in PBS with 5% FCS

OPD Substrate: 1 mg/ml O-Phenylendiamin in Substrate Buffer, 1 µl/ml H_2O_2

Substrate Buffer: 0,2 M NaH_2PO_4, 0,1 M Na_3-citrat; pH 5,0

ELISA was carried out in 96 well plate (MaxiSorb™ Immunoplates, Nunc, Wiesbaden, Germany).

ELISA-washer (Dynatech, Denkendorf, Germany)

ELISA-Reader MR 5000 (Dynatech, Denkendorf, Germany)

2.14.6 Immunohistology

1/3 of spleens were embedded in Tissue-Tek O.C.T. compound (Sakura, Zoeterwounde, The Netherlands), snap-frozen in liquid nitrogen and stored at –20°C. Cryostat sections of 7µm were cut by cryotom Frigocut 2800 (Reichert-Jung, Nussloch, Germany), air dried for 2 h and fixed in acetone (2 min at –20°C). After thawing and rehydration, slides were stained at RT. To inhibit possible unspecific staining sections were treated for 30 min with anti-mouse FcR Abs in BSA/PBS at 4°C. In case of staining with goat-anti-rabbit Abs conjugated with peroxidase (PO), endogenous peroxidase was blocked for 20 min at RT. Sections were then stained with Abs described above for 1 h at RT. After washing biotinylated Abs were revealed using streptavidine (SA): fluorescent SA-Cy™3 (Dianova, Hamburg, Germany) or SA-AP (Sigma-Aldrich, Deisenhofen, Germany) for 30 min. For developing of PO or AP color

sections were incubated 10 min at the RT with DAB solution (Sigma-Aldrich, Deisenhofen, Germany) or Fast Red solution (Sigma-Aldrich, Deisenhofen, Germany), respectively. Non-fluorescent staining was conducted using TBS as a blocking and washing buffer, fluorescent with PBS/BSA buffer. After staining slides were dried, mounted with Neo-Mount (Merck, Darmstadt, Germany) and analyzed using an optical or confocal microscope.

PBS/BSA buffer:	0.05% BSA in PBS, pH 7.2
TBS	10mM Tris-HCl, 150mM NaCl, 0.05% Tween20, pH 7.6
Peroxidase blocking buffer:	5% NaN_2, 3% H_2O_2 in PBS
DAB	3-3' diaminobenzidine tetrahydrochloride, a chromogen, substrate for a peroxidase, provides organic solvent-stable permanent chromogen depositions (brown)
Fast Red	Chromogen, substrate for alkaline phosphatase (AP), red

Four-color confocal microscopy (AMCA, FITC, Cy3/Alexa568, APC/Cy5/CyChrome) of cryosections was performed using a MRC 1024 UV confocal microscope (Bio-Rad, München, Germany) equipped with two lasers (Argon-Ion: 353/361 nm, Krypton-Argon: 488 nm, 568 nm, 647 nm). To avoid overlapping emissions, fluorescent dyes were selectively excited in two series and fluorescence of single channels was measured by photon counting. Images were processed with Confocal Assistant 4.02, ImagePro 4.5 (Media Cybernetics, Gleichen, Germany) and Adobe Photoshop 7.

2.15 Polyclonal Abs treatment

To neutralize CCL2 (JE) produced at the early stage of *Listeria* infection, mouse JE/CCL2 affinity purified polyclonal Abs (R&D Systems, Wiesbaden, Germany) were used. 10µg of Abs was injected iv in 100 µl of sterile PBS, 1 h before bacterial infection. After 1 h animals were injected iv with $2x10^3$ or $5x10^5$ CFU of *Listeria monocytogenes*, then 0, 1, 2, 3, 4, 5 days or 0, 2, 4, 6, 24 h PI, respectively, mice were sacrificed, spleens removed and prepared for RT-PCR, histology or flow cytometry as described above.

3 Results

Upon interaction with the host, *Listeria monocytogenes* induces strong cellular responses. This is on the one hand due to the host cell receptors such as scavenger receptors, responsible for phagocytosis or the toll like receptors that interact with bacterial components (Edelson and Unanue 2002). On the other hand, it is known, that virulence factors can also induce specific signal cascades upon encountering the host cell surface. The spectrum of such events reaches from rearrangement of the cytoskeleton induced by the internalins up to induction of apoptosis of dendritic cells (DCs) by listeriolysin (Guzman et al. 1996). In this context, it is well established that *L. monocytogenes* induces the secretion of several cytokines in macrophages (Unanue 1996;Kaufmann and Kaplan 1996;Edelson and Unanue 2000) and upregulation of cell adhesion molecules on endothelial cells (Drevets 1997). Many of such reactions of the host cell involve the activation of the transcription factor NFκB (Whitley et al. 1994;Drevets 1997;Kuhn et al. 1999;Kayal et al. 2002) but other pathways are also known to be induced (Tang et al. 1996;Sibelius et al. 1996).

Despite of the extensive studies, little is known about the early events caused by *L. monocytogenes* upon macrophage encounter. Since it is generally accepted that these are the first cells that remove the bacteria from circulation, such interactions have very important after-effects for the further development of the immune response.

Investigations in this direction are impaired because of the difficulty to isolate sufficient numbers of infected cells for analysis. To avoid such problems, here first an established macrophage cell line J774 was used for global analysis of genes induced after infection by *L. monocytogenes in vitro*. Subsequently, these results were confirmed in other macrophage subsets *in vitro*. Then the effect of *L. monocytogenes* on selected genes *in vivo* was studied. Cells were isolated from infected mice, based on their adherence or sorted and the expression of the genes was analyzed.

Finally, the function of the chemokine dominating during the early stage of infection was investigated. Antibodies against this protein were injected into the mice before iv infection with *Listeria* and the host immune responses were followed.

3.1 L. monocytogenes infection in vitro

3.1.1 Analysis of gene expression in the host cell J774 after infection

The macrophage line J774 was selected first to be tested, since infection of this cell line with *L. monocytogenes* represents a commonly used infection model and is routinely used in our group.

3.1.1.1 Establishing of the optimal conditions for infection

Optimized *in vitro* infection of the macrophages required the knowledge of the susceptibility of these cells and their sensitivity to the bacterial invasion. Since macrophages are a very heterogeneous population it is suitable to test the parameters, like optimal multiplicity of infection (MOI) and survival of cells, each time before working with a different subset.

In order to check the optimal MOI, the macrophage-like J774 cell line was infected with different numbers of *L. monocytogenes* EGDe and a variant strain deleted for LLO; Δhly. LLO is the listerial virulence factor known to induce signaling cascades in host cells. MOIs 5, 10, 50 and 100 were used in this experiment. After 24 h the supernatants from infected cells were analyzed for activities induced due to the infection. The secretion of IL-6, the pro-inflammatory cytokine usually produced by activated macrophages was used as a readout system.

In case of infection with the wild type EGDe, MOI 5 already induced IL-6 secretion, measured by an IL-6 specific ELISA, but its maximal level was reached at MOI 10 (Figure 3.1). Higher bacteria numbers did not change the cytokine production, but caused massive macrophage death (data not shown). For Δhly maximum level of IL-6 was reached at MOI 50, but cell death was then extremely high. MOI 10 was therefore chosen for both bacterial strains as optimal for further experiments.

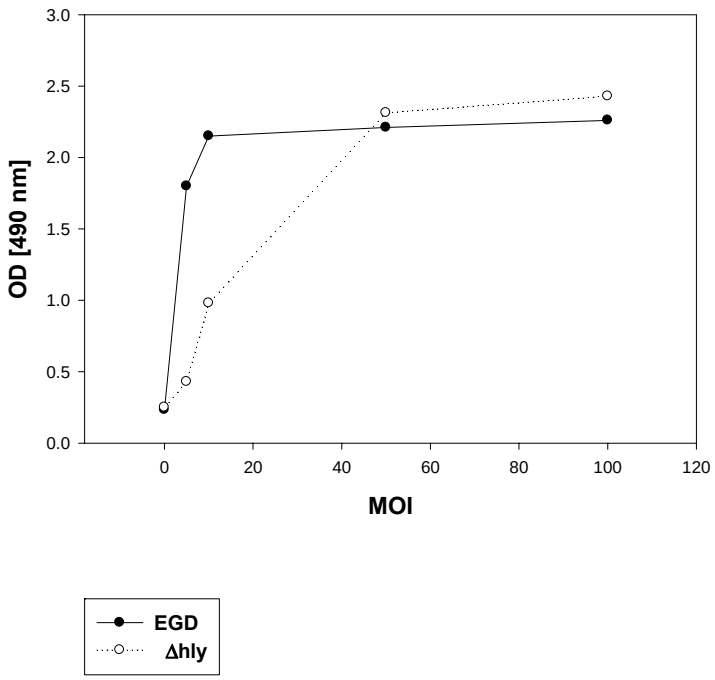

Figure 3.1 IL-6 production by *L. monocytogenes* infected J774 macrophages. J774 cells were infected with the different MOIs of bacteria. After 24 h cell culture medium was analyzed for IL-6 content. To obtain comparable results, care was taken that the determination of the IL-6 content indicated by the OD of the colorimetric reaction was performed on the same plates for the various experimental conditions.

A very important factor for the cell infection is also the time course of bacterial treatment. In order to get the proper information about expression of genes regulated after infection, isolated RNA should not be degraded. Degradation of RNA could be the consequence of the apoptotic cell death due to the high bacterial burden or too extended time of infection.

To test this, J774 macrophages were infected with *L. monocytogenes* EGDe and Δhly at MOI 10 and after different time points (2, 4, 6 and 24 h) the number of intracellular bacteria was estimated. For both *Listeria* strains intracellular bacterial burden increased until 6 h PI and then rapidly decreased (Figure 3.2). It became obvious that this is due to the death of macrophages or pores in their cell surface, and subsequent contact of the intracellular bacteria with the gentamycin in the culture medium. To avoid RNA degradation, 2 and 6 h PI were chosen as time points for further experiments.

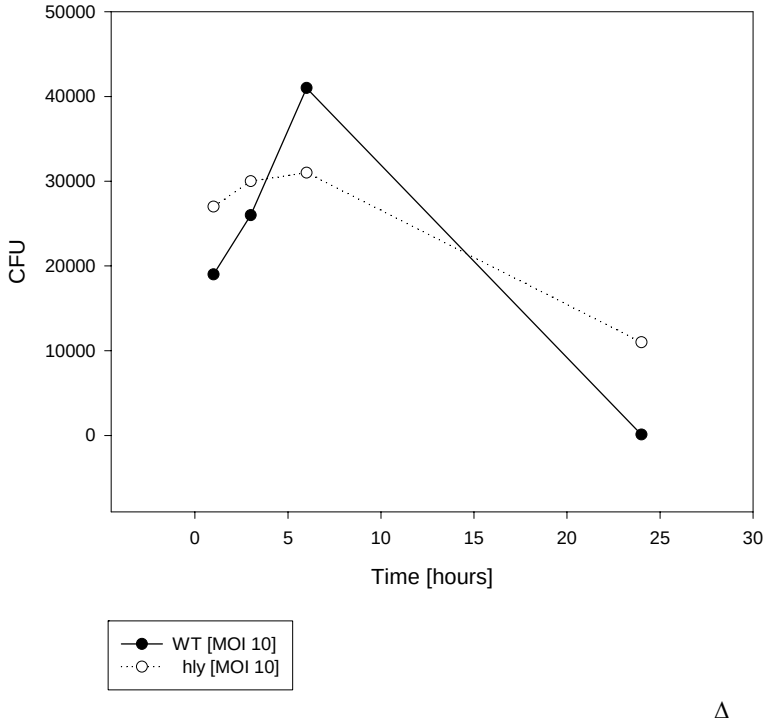

Figure 3.2 Number of intracellular bacteria after J774 infection. Lysates of infected cells were prepared by washing cells with PBS and then adding 500µl of 0.1% Triton X-100 in H_2O per well. Cells were the incubated for 30 min at room temperature (RT). To determine the number of the viable intracellular *Listeria*, serial dilutions of the lysates were plated on the BHI agar plates.

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3.1.1.2 Analysis of gene expression in J774 after *L. monocytogenes* infection

The most global and efficient approach to establish the gene expression pattern in cells is the use of expression arrays (Chalifour et al. 1994;DeRisi et al. 1996). Here two types of arrays were used, based on different principles.

Macro expression array

In the first approach, cDNA arrays interrogating approximately 1200 genes were employed. J774 cells were infected with the two *L. monocytogenes* strains, EGDe and Δhly, cells were harvested 2 and 6 h PI, cDNA prepared and analyzed on the macroarray. Table 3.1 displays genes that were found to be at least 3-fold regulated. Mainly pro-inflammatory cytokines and chemokines showed strong upregulation. In addition, a few receptors known to be involved in host reactions, as well as two enzymes with immunomodulatory functions. Interestingly, most of the genes such as IFNβ, ICE, CXCL10 (IP-10) and IFNα/β receptor required listeriolysin to be strongly regulated, since *L. monocytogenes* Δhly did not or only poorly induce these genes (Table 3.1).

Fold Change (control/infected cells)				Gene name	GeneBank
2h		6h			
EGD	Δhly	EGD	Δhly		
Chemokines					
-	-	3.7	-	XCL1 (Lptn)	U15607
-	2.8	4.1	3.2	CCL1 (JE)	M17957
4.1	2.8	1.8	1.8	CCL2 (MCP-1)	J04467
5.3	3.5	6.2	4.5	CCL3 (MIP-1 alpha)	X12531
5.7	3.4	5.2	4.7	CCL4 (MIP-1 beta)	M35590
9.8	4.9	3.1	4	CCL7 (MCP- 3)	S71251
-	-	-	-	CCL9 (MIP-1 gamma)	U49513
-	-	-	-	CCL21 (6Ckine)	AF001980
-	-	-	-	CCL25 (TARC)	AJ249480
29	18	12	19	CXCL2 (MIP-2)	X53798
-	-	-	-	CXCL9 (MIG)	M34815
10	1.5	7	2.4	CXCL10 (IP-10)	M86829
Cytokines					
19.2	2.6	1.1	1.8	IFN alpha family gene B	L38698
53	4.2	8.3	1.3	IFN beta	K00020
0.9	0.3	10.3	7.4	IFN gamma	K00083
2.7	1.6	6.3	4.9	IL-1R antagonist	M74294
1.5	1.4	1.5	3.3	IL-1 alpha	X01450
7.3	8.4	33	10	IL-1 beta	M15131
10.5	1.5	2.8	1.2	IL-2	X01772
87	15	35	23	IL-6	X06203
-	-	-	-	IL-12 alpha	M86672
1	1.8	7.1	2.4	IL-12 beta	M86671
-	-	-	-	IL-15	U14332
-	-	-	-	IL-16	AF006001
-	-	-	-	IL-18	D49949
Receptors					
55	34	-	-	CD3 zeta	M33158
79	-	5.3	17.3	CD30	U25416
3.2	-	5.6	-	CD40	M83312
3.5	-	4.3	4	CD83	AF001041
11	-	5.3	1.9	CCR7	L31580
3	-	-	-	IFN alpha/beta R	M89641

Table 3.1 Regulation of genes after infection of macrophage cell line J774 with *L. monocytogenes* (Atlas mouse 1.2 Array, Clontech). RNA was extracted from $1x10^7$ infected and was used as a template for synthesis of ^{32}P-labeled cDNA probes. Probes were hybridized to the gene array membranes containing various mouse cDNAs (Atlas mouse 1 and 2 Array, Clontech). Membranes were then exposed to Phosphor imaging Screens (Fuji) and developed using a Fuji Bas 2500 16 bit image analysis system. Analysis was carried out in duplicates and evaluated with Array-Vision softwear 5.1 (Imaging Research).

Fold Change (control/infected cells)			
2h	**6h**	**Gene Symbol**	**GeneBank**
Chemokines			
-	-	XCL1 (Lymphotactin)	U15607
-	-	CCL1 (JE)	M23501
2.3	3.3	CCL2 (MCP-1)	J04467
2.2	3.2	CCL3 (MIP-1 alpha)	X12531
4.1	6.7	CCL4 (MIP-1 beta)	M35590
-	20	CCL5 (RANTES)	AF065947
7.6	21	CCL7 (MCP-3)	S71251
-	11	CCL12 (MCP-5)	U50712
-	-	CCL19 (MIP-3 beta)	AW120505
-	-	CCL21 (6Ckine)	AF001980
-	-	CCL25 (TECK)	AJ249480
-	-	CCL27 (CTACK)	AA672499
-	-	CXCL1 (GRO alpha)	J04596
6.6	12	CXCL2 (GRO beta)	X53798
-	-	CXCL5 (ENA-78)	U27267
-	-	CXCL9 (MIG)	M34815
5.1	6.5	CXCL10 (IP-10)	M86829
-	-	CXCL12 (SDF-1)	L12029
-	-	CX3CL1 (Fractalkine)	U92565
Cytokines			
21	79	IFN beta	K00020
-	6.5	IL-1alpha	X01450
9.5	63	IL-1beta	M15131
-	30	IL-1R antagonist	M74294
-	-	IL-2	X01772
-	-	IL-4	X03532
-	49	IL-6	X06203
-	-	IL-7	X07962
-	-	IL-9	M30136
-	-	IL-10	M37897
		IL-11	U03421
-	-	IL-12 beta	M86671
-	-	IL-17D	AI835126
-	8.5	GM-CSF	X03020

Table 3.2 Regulation of genes after infection of macrophage cell line J774 with *L. monocytogenes* (Affymetrix Array; Mu 11K). RNA was isolated from $1x10^7$ infected cells and double stranded cDNA was prepared. An *in vitro* transcription reaction was performed to produce biotin-labelled cRNA, the hybridization cocktail was prepared and hybridized to the oligonucleotide spots on the micro array (Mu 11K – 11,000 murine genes and EST clusters) for 16 hrs at 45^0C. Data were then analyzed by GeneChip software provided by the Affymetrix GeneChip. Data from two independent infections were compared. Expression levels differing more than two-fold were considered significant.

Micro expression arrays

To confirm and extend the data obtained using macroarrays, genes regulated after listerial infection were analyzed using microarrays, which contained oligonucleotides representative for approximately 1100 genes and ESTs. In this case only EGDe bacteria were used for infection, since the influence of LLO could be tested on selected genes.

After normalization of the double determinations, 20 and 100 genes were significantly regulated 2 and 6 h PI, respectively. All genes regulated at least 2 fold under these circumstances are presented in Addendum 1. Since the rational of these experiments was to find regulated genes that influence the immune reactions against *L. monocytogenes*, some of these genes were considered as genes of interest and displayed in Table 3.2. As confirmation of the initial data set obtained with the macroarrays, a similar induction pattern of inflammatory cytokines and chemokines was observed.

3.1.1.3 The confirmation of gene regulation by Real-Time RT-PCR

Chemokines are considered the most influential molecules for the initiation of the immune reactions, therefore most experiments in this work focused on these molecules. Since the induction of IFNβ observed by the expression arrays was unexpected, it was also included in the analysis.

To corroborate the array data and test the general applicability of this approach, chemokine expression of *Listeria* infected J774 cells was quantified by Real-Time RT-PCR. Cells were infected like described above, after 2 and 6 h RNA was isolated and cDNA prepared. Optimal forward and reverse primer concentrations for reaction were established using the primer matrix i.e. by testing primer pairs at different concentrations. All results obtained using Real-Time RT-PCR were normalized to the housekeeping gene RPS9.

These experiments confirmed the regulation of the chemokines and IFNβ observed using expression arrays (Figure 3.3). Already 2 h PI IFNβ and CXCL2 were very strongly upregulated, reaching a 20 fold increase of mRNA level. Most other chemokines required 6 h for maximal induction of expression.

3.1.2 Induction of the inflammatory chemokines by *L. monocytogenes* in Raw264.7 and bone marrow derived macrophages

Macrophages are an extremely heterogeneous population depending on their anatomical location and activation state (Rutherford and Schook 1992). To check the gene expression pattern induced by *Listeria*, two additional subsets of macrophages were analyzed: the cell line Raw264.7 and bone marrow derived macrophages (BMDM).

3.1.2.1 Optimization of the infection conditions for Raw264.7 and BMDM

The optimal MOI for the infection of Raw264.7 and BMDM was established using the same strategies as described for J774. Cells were infected with different MOIs of *L. monocytogenes* and the production of IL-6 was measured by ELISA after 24 h. Interestingly BMDM turn out to be the most resistant cells for the bacterial infection, since only at MOI 50 they upregulate the IL-6 production. For the monocyte-macrophage cell line Raw 264.7 MOI 25 was sufficient (data not shown).

3.1.2.2 Real-Time RT-PCR for inflammatory chemokines in different types of macrophages

After infection of both macrophage subsets, RNA was isolated and Real-Time RT-PCR was conducted after reverse transcription. Raw264.7 and BMDM represent different types of macrophages; therefore, one would expect differences in the gene expression pattern induced by the listerial infection. This turned out to be the case (Figure 3.3). Genes encoding CCL4, CCL12 and CXCL10 were upregulated in Raw264.7 and BMDM like in J774. In contrast, other chemokines were only weakly induced in Raw264.7 (CCL5 and CCL7) or in BMDM (CCL3), while CCL2 and CXCL2 were hardly regulated in either Raw264.7 or BMDM. Interestingly IFNβ was induced in all of these macrophages.

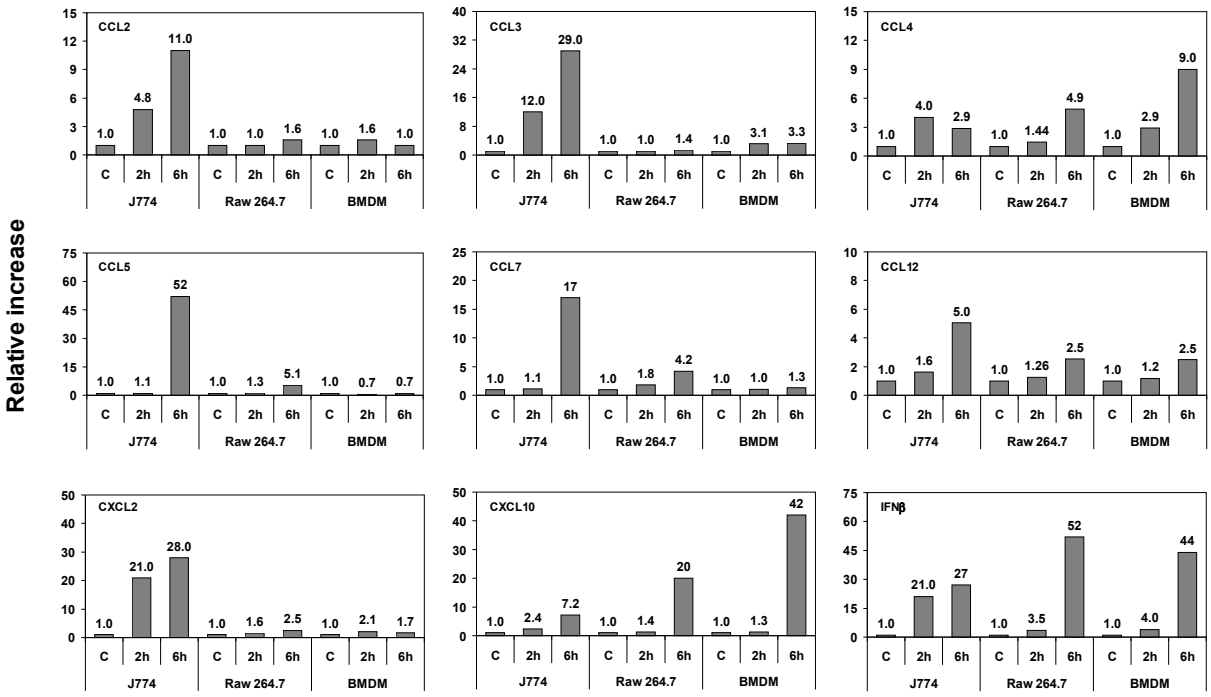


Figure 3.3 Induction of inflammatory chemokine genes in macrophages by *L. monocytogenes* infection *in vitro*. Macrophage cell lines J774, Raw264.7 or macrophages generated from bone marrow cells (BMDM) were infected and RNA from samples taken 2 and 6 hrs PI were analyzed by Real -Time PCR. Results are expressed as the fold increase of mRNA relative to the uninfected control. Infection experiments were carried out at least twice, with similar results.

3.2 Induction of cytokines and chemokines in BALB/c mice during the initial phase of L. monocytogenes infection in vivo

It is obvious that experiments *in vitro* are just a simplification of the complex reactions taking part in live organism. Thus, the *in vitro* studies in this work should be considered an establishment of principle regulatory phenomena. However, they gave essential clues for the direction of further analysis *in vivo*.

Thus, based on the *in vitro* findings, the host reactions induced by *L. monocytogenes* during the initial phase of infection were examined. Since *in vitro* the majority of upregulated genes at the early time points were pro-inflammatory cytokines and chemokines, induction of the same genes were studied *in vivo*. BALB/c mice were employed first for such experiments, as the *in vitro* conditions were established with cells of BALB/c origin. This commonly used mouse strain is very susceptible to *Listeria* infection and the LD_{50} is $4x10^3$. They are also known for their Th2 directed responses in many infections, although during *L. monocytogenes* infection Th1 responses dominate in such mice.

3.2.1 Infection of BALB/c mice with a high dose of *L. monocytogenes*

During the *in vitro* infection a high *Listeria* number was used to ensure optimal interaction between the host cell and bacteria. In living organism such conditions are difficult to reach, since only a few macrophages encounter bacteria during the initial phase of infection and the load of pathogen will be much lower. In order to at least approximate similar conditions during the *in vivo* study, a high dose of *L. monocytogenes* was used. Such a dose should facilitate the analysis of early events during the course of *Listeria* infection. It should increase signals induced by bacteria in macrophage populations of the spleen and also allow the detection of bacteria in this organ.

Mice were infected iv with $5x10^5$ CFU of *Listeria* and after 0, 2, 4, 6 and 24 h mice were sacrificed, spleens removed and spleen cells isolated for further analysis.

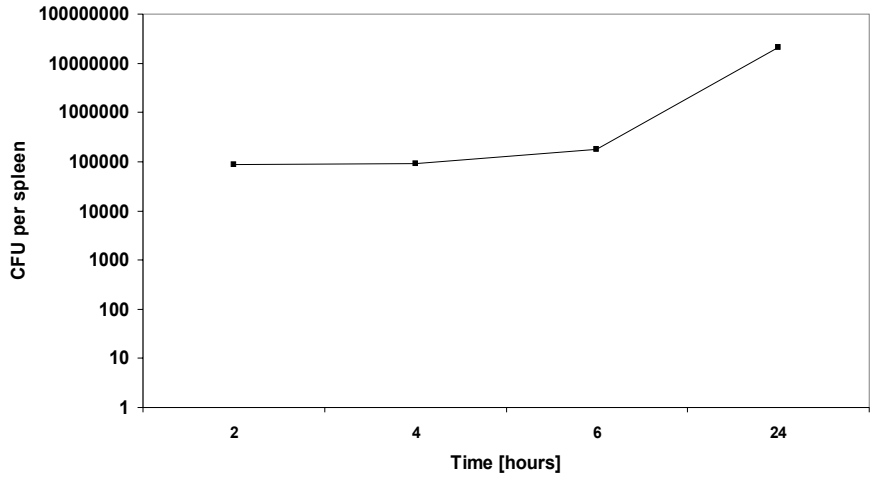


Figure 3.4 Growth curve of *L. monocytogenes* in the spleen of the iv infected BALB/c mice. Female mice were infected intravenously with $5x10^5$ (a high dose infection) CFU of *L. monocytogenes.* Number of injected *Listeria* was confirmed by plating an aliquot of the inoculum on BHI plates. After 0, 2, 4, 6 and 24 hours spleens were removed, parts of the organs homogenized in PBS supplemented with 0.2% NP-40 and plated on BHI agar. After overnight incubation at 37^oC CFU of bacteria per spleen was estimated.

3.2.1.1 Estimation of *L. monocytogenes* load in the spleen of infected BALB/c mice

To measure efficiency of the infection, spleen fragments were homogenized and plated in serial dilutions on the BHI agar plates. After overnight incubation at 37^{o}C the number of bacteria per organ was estimated (Figure 3.4). It is obvious, that after a high dose injection mice cannot control the infection anymore. At the time of sacrifice, 24 h PI the mice were severely affected and looked ill.

3.2.1.2 Regulation of the expression of the genes encoding inflammatory cytokines and chemokines

Data obtained by *in vitro* experiments showed upregulation of numerous pro-inflammatory cytokine and chemokine genes in infected macrophages. In order to check the relevance of these findings during *in vivo* infection, spleen cells were prepared from infected animals at the different time points mentioned above. To follow gene expression in macrophages, the spleen cell population was enriched in these cells by adherence to tissue culture plates (adherent cells). This population consisted mainly of macrophages and DCs, but also B cells were found (data not shown). Then RNA was isolated and transcribed into cDNA, which was used for subsequent RT-PCR amplification. Regulation of investigated genes was then compared between adherent and total spleen cell populations.

First, expression of the genes coding for cytokines known to be induced in the early phase of *L. monocytogenes* infection was tested. While *in vitro* many of the cytokines and chemokines were produced by the same macrophage, differential expression patterns probably due to cell-cell interactions became apparent *in vivo.* Obviously, under such conditions cytokines were sequentially activated. By 4 h PI, IL-1β and TNFα could be detected mainly in the adherent cell population. Their expression remained high throughout time points tested (data not shown). Surprisingly, IL-6 that was strongly induced during the *in vitro* infection experiments (Table 3.1 and 3.2) could hardly be detected under *in vivo* conditions (Figure 3.5). In agreement with previous reports (Unanue 1997;Vazquez-Boland et al. 2001), we could detect IL-12p40, IFNγ and IL-10 expression in the macrophage enriched adherent population 4 h post infection. The inducible NO synthetase, an enzyme usually activated during inflammatory responses, was also analyzed in this study. Its expression could only be detected in the adherent spleen cell population 24 h PI. Since iNOS is mainly expressed in activated macrophages (MacMicking et al. 1997) the exclusive appearance of iNOS mRNA in the

adherent population can be taken as indicator for the enrichment of macrophages in this cell population.

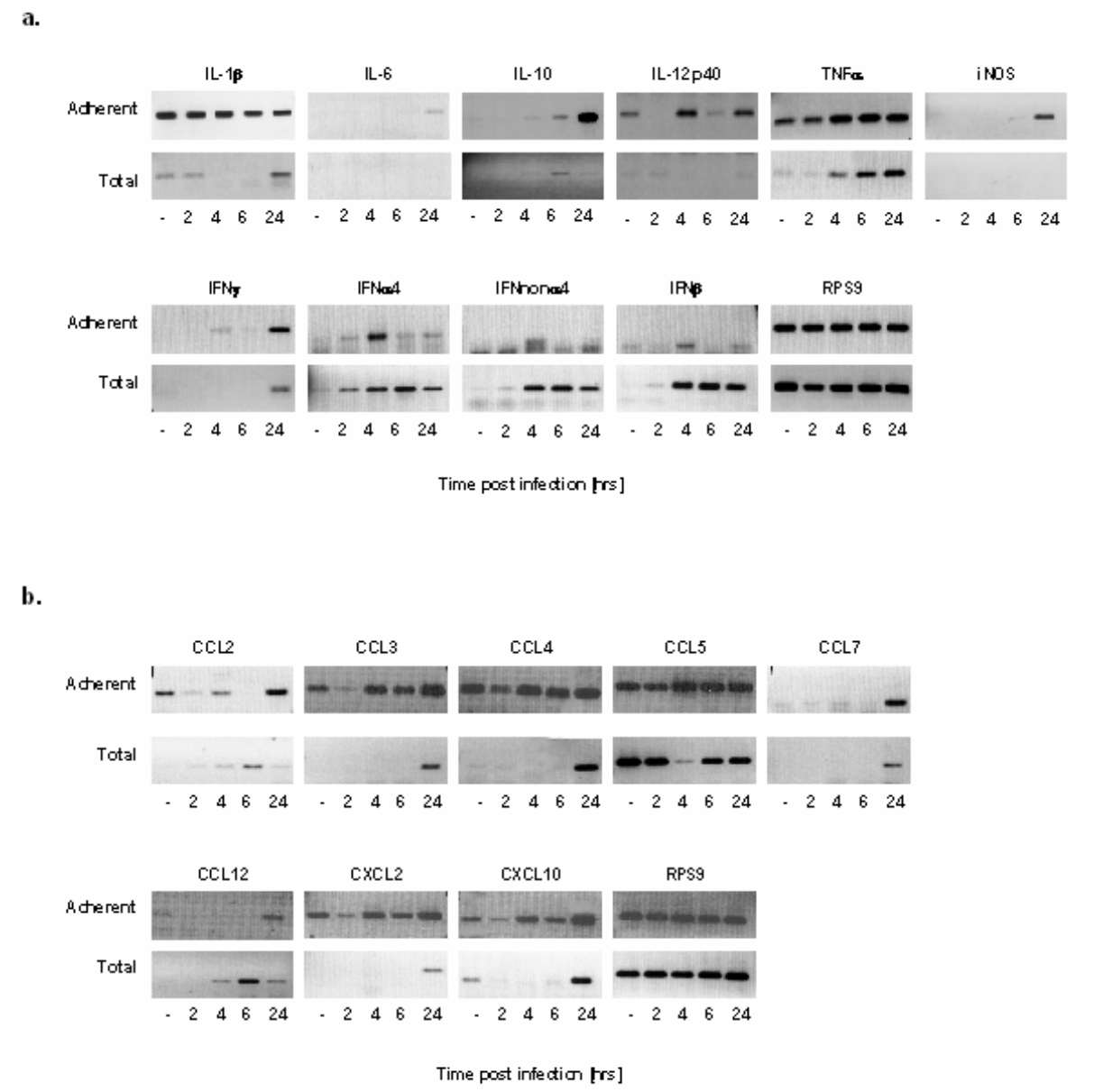


Figure 3.5 RT-PCR analysis of the cytokine and chemokine genes regulation in the spleen upon *Listeria* infection.

Since IFNβ was induced in all types of macrophages *in vitro*, we examined by RT-PCR the induction of type I interferons, namely IFNβ, IFNα4 and all non α4 IFNs. Although *in vivo* these cytokines were quickly induced after infection with *L. monocytogenes* and remained detectable during 24 h period, their expression was mainly restricted to the total spleen cell population. In the macrophages enriched adherent population only a weak signal could be detected 4 h PI. This indicates that cells other than macrophages and DCs are the main contributors of this cytokine.

Testing both populations for inflammatory chemokines revealed that production was mainly restricted to the adherent cell population. Except CCL5, which was constitutively expressed, all chemokine genes tested seemed to be upregulated during the course of infection as soon as 4 h PI. Interestingly CCL12 seems to be upregulated earlier in cells not present in the adherent cell population while in adherent cells it appears late, at 24 h PI.

Obviously in the early stage of infection, chemokines produced by the macrophages enriched population are involved in the attraction of other phagocytes and thus are most likely responsible for the severe remodeling of the spleen that accompanies *L. monocytogenes* infection (see below). Since the expression of such inflammatory chemokines was mainly restricted to the adherent spleen cells (Figure 3.5b), subsequent quantitative analysis of regulation of these molecules was performed only on this population. For this measurement Real-Time RT-PCR was used.

In general the qualitative patterns were confirmed by the quantitative determination. Some discrepancies were observed. They might be explained by assumption that the plateau of the particular reaction is reached earlier during the amplification compared to others.

Quantitation of mRNA revealed that of the inflammatory chemokines investigated only CCL2 and CCL7 showed a significant upregulation 4 h PI (Figure 3.6). The dramatic upregulation of CCL2 (1500 fold) was transient, declining to almost background level by 24 h PI. In contrast to the early induction of those two chemokines, CCL3, CCL4, CCL12, CXCL2 and CXCL10 exhibited a late expression (‘late chemokines’) with the highest level at 24 h PI. Among these chemokines, CXCL10 showed the most dramatic induction (1600 fold). Results regarding IFNβ regulation were also confirmed. In the macrophages enriched population, no increase in IFNβ mRNA could be detected.

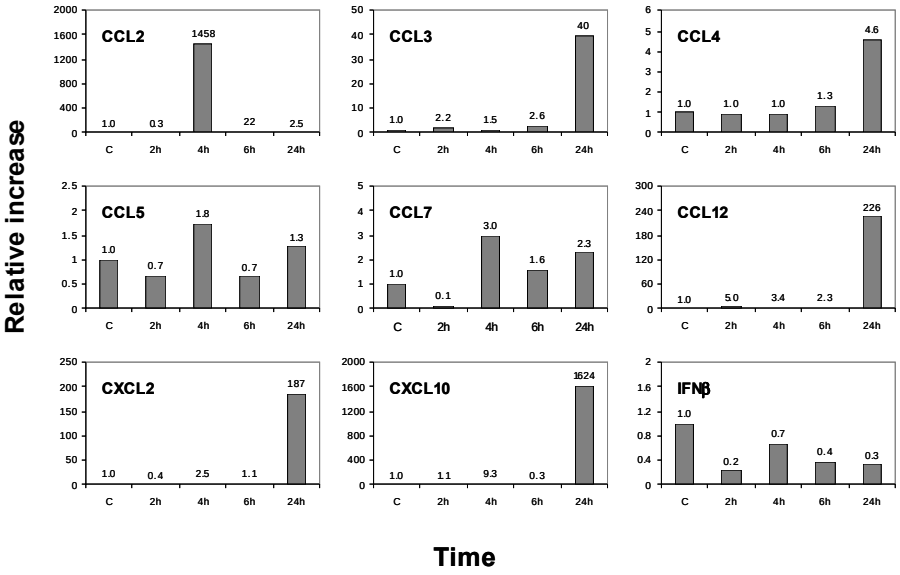


Figure 3.6 Quantitation of chemokine gene expression in macrophages from the spleen of mice infection with a high dose of *L. monocytogenes*. Mice were infected intravenously with 100xLD50 of *L. monocytogenes* and specific RNA was quantified by Real-Time PCR at the indicated time points from the adherent spleen cell population. Results are expressed as the fold increase in comparison to the uninfected control. The experiment was performed independently at least twice and identical results were obtained.

3.2.1.3 Impact of *L. monocytogenes* infection on the architecture of the spleen

With such an upregulation it is to be expected that the architecture of the spleen should be severely changed. Cells should be migrating along chemokine gradients established by cells affected by the bacteria. They should then also produce inflammatory molecules, which in turn might stimulate other cells. The complexity of such events can be observed using immunohistological methods on spleen sections from infected animals. Staining with the appropriate antibodies should allow the identification of the cell populations induced and the kinetics of migration of these cells.

Since it is well established that after *iv* administration, bacteria are quickly removed from the circulation by marginal zone macrophages, surrounding the lymphoid follicles in the spleen, these cells were examined first. There are two types of macrophages in this location that can be distinguished by cell surface markers - MOMA-1^+ and ERTR-9^+ in immunohistology (Figure 3.8 and 3.9). Especially interesting for this study are macrophages bearing the ERTR-

9 marker. These cells possess extensive dendrites reaching each other and embracing other kinds of cells, thus forming a network in the area of the marginal zone (Figure 3.7).

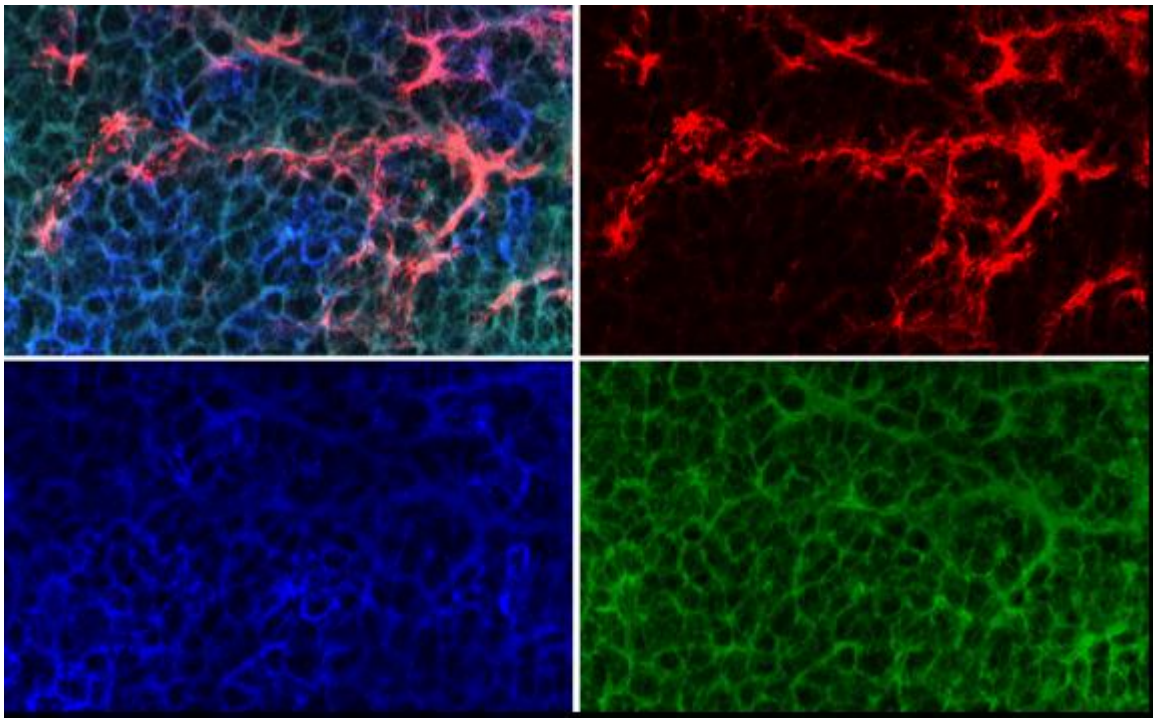
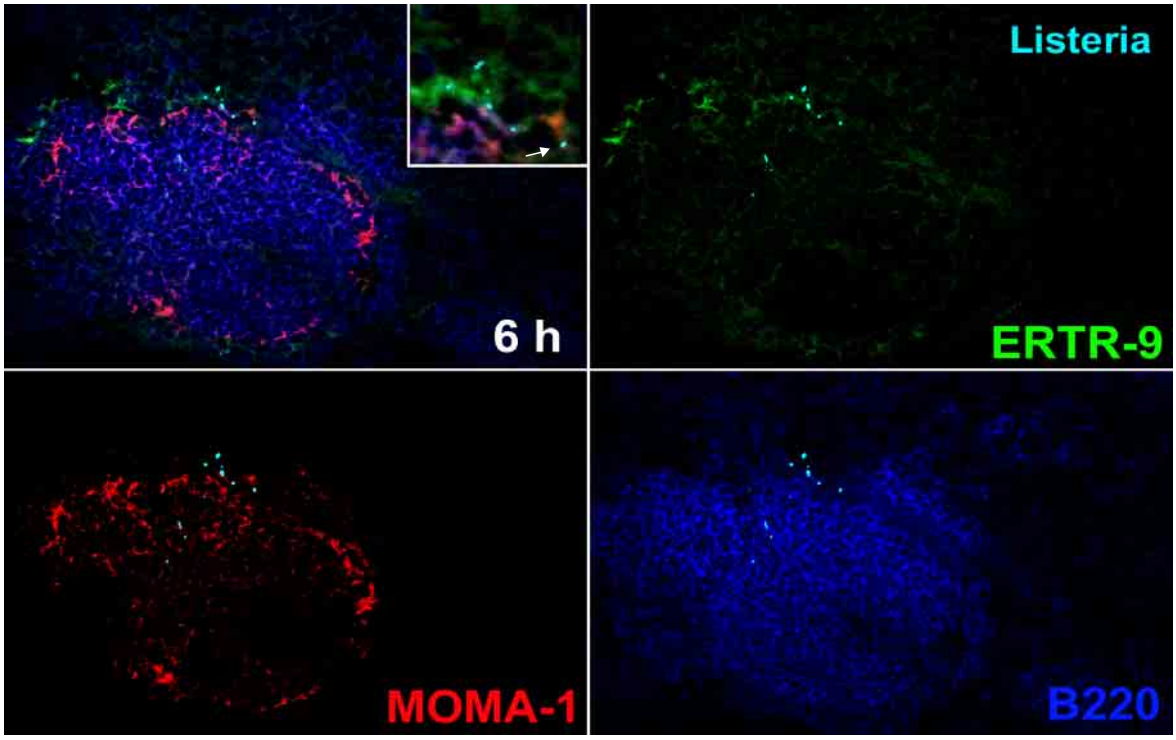


Figure 3.7 Network of the ERTR-9$^+$ cells. CD3$^+$, B220$^+$

Staining of spleen sections from infected mice with anti-ERTR-9 and anti-MOMA-1 Abs revealed a different behavior of these macrophages. Only macrophages of the ERTR-9 type were found to contain bacteria 6 as well as 24 h PI (Figure 3.8). Thus, these cells are most likely the source of chemokines that are produced in the initial phase of the infection (Figure 3.6).

The macrophages seem to have also a totally diverse function. ERTR-9$^+$ cells form clusters around infection foci by 24 h PI (Figure 3.8), while MOMA-1$^+$ cells migrate into B cell follicle (Figure 3.9). Interestingly, the latter cell population seemed to be activated without direct contact with *Listeria*. The migration could be the result of chemokines produced inside a B cell follicle or upregulation of an appropriate chemokine receptor in MOMA-1$^+$ cells during *Listeria* infection. However, the reason for this migration and also the function of these macrophages are not clear and need to be investigated further.

a.



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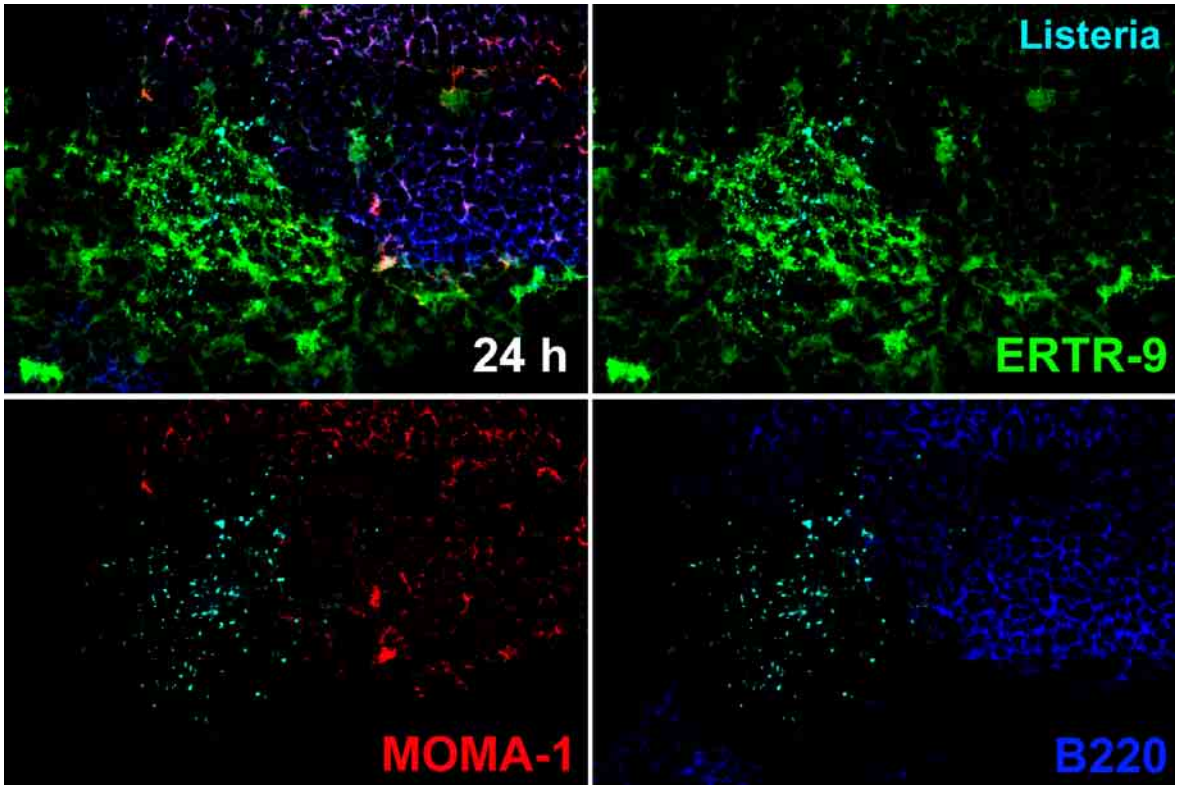


Figure 3.8 ERTR-9^{+} macrophages are the only cells involved in the uptake of *Listeria* from the blood.

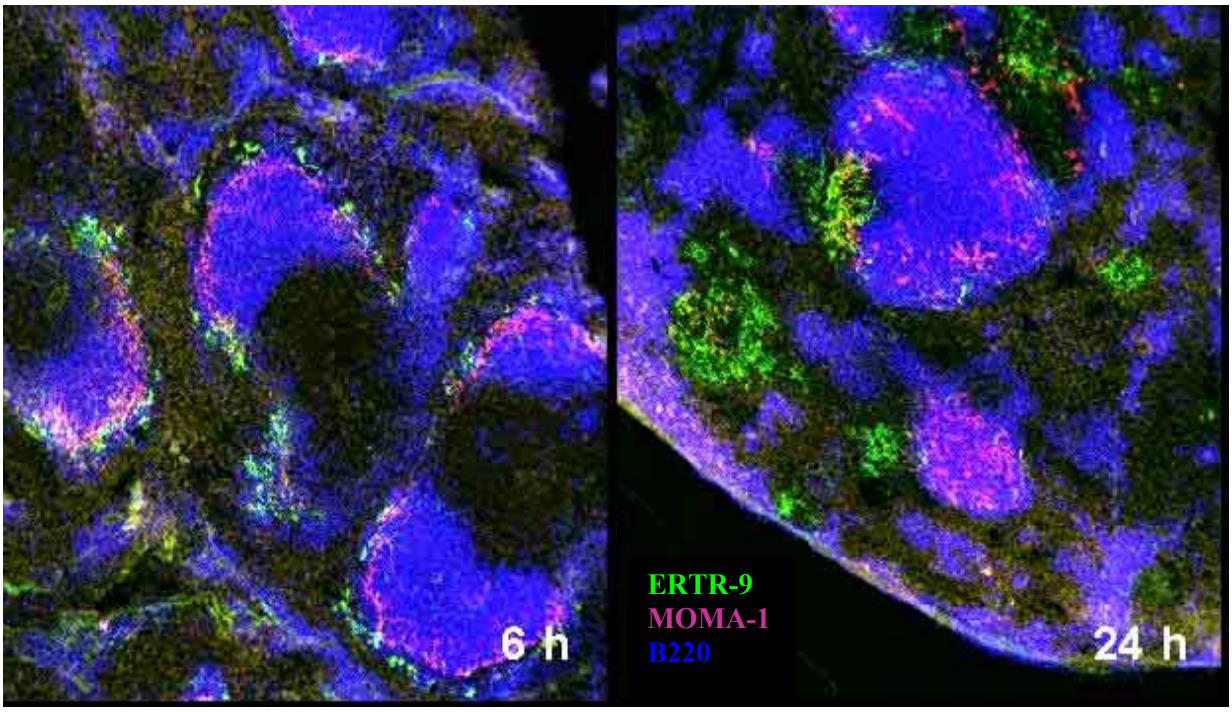


Figure 3.9 Clustering of ERTR-9$^+$ cells around infection foci and puzzling migration of MOMA-1$^+$ cells inside the follicle.

To follow changes in the macrophage relocations after *L. monocytogenes* infection, sections were stained with anti-Mac-1 Abs. This surface marker is present on the majority of macrophages in the spleen localized in T cell areas, in the marginal zone as well as scattered in the red pulp. After the administration of a high dose of *Listeria* by the *iv* route, arrangement of Mac-1$^+$ cells underwent drastic changes. Migration of these cells could already be observed by 4 h PI (Figure 3.10), and becomes more obvious by 24 h PI. By then, Mac-1$^+$ cells, of which a few contained bacteria, are almost exclusively located within clusters in which B220$^+$ cells with dendritic shape and a few T cells can also be found (Figure 3.11). Since ERTR-9$^+$ macrophages were also found in these clusters, it is likely that these cells also express the Mac-1 marker. This could be due to upregulation of this marker upon *Listeria* infection. In contrast, Mac-1$^+$ cells were never found inside the follicle. Thus MOMA-1$^+$ cells remain negative for this marker.

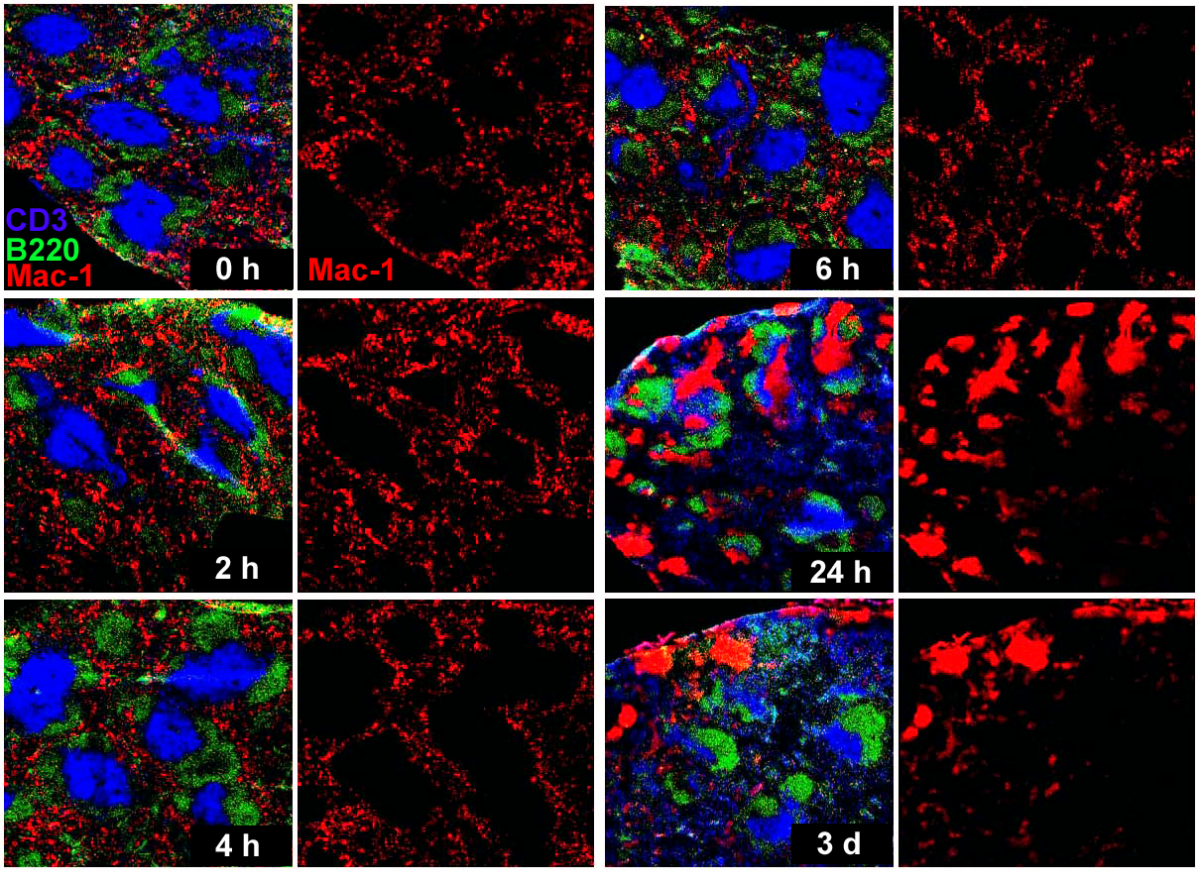


Figure 3.10 Relocation of the Mac-1$^+$ macrophages in the spleen of infected mice. Spleens were removed and cut as described above. Cryosections were stained with anti-Mac-1, anti-CD3 and anti-B220 Abs and analyzed using confocal microscope. Migration of the Mac-1$^+$ cells becomes obvious after 4 h PI and results in cell clustering after 24 h. For comparison staining of spleens infected with a low dose infection ($2x10^3$) was employed. The same rearrangement of the Mac-1$^+$ cells in the spleens at 24 h with a high dose and at 3 days after a low dose infection.

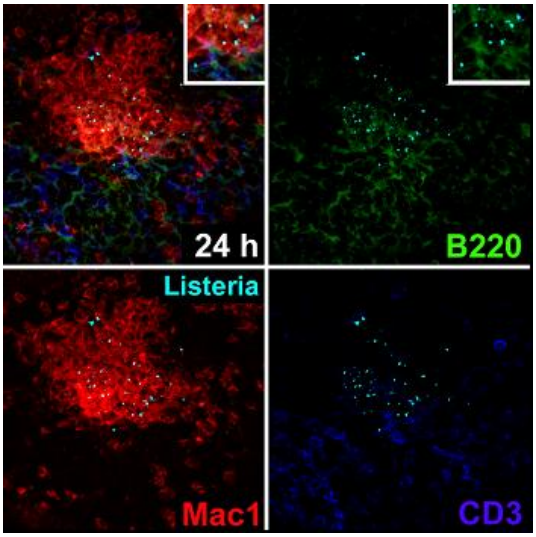


Figure 3.11 Clusters of Mac-1$^+$ cells around trapped *Listeria* at 24 h PI. Spleens from infected animals 24 h PI were stained with anti-Mac-1, anti-B220 and anti-*Listeria* Abs. Mac-1$^+$ macrophages together with a few CD3$^+$ T cells surround bacteria.

So far, *Listeria* were never detected in CD11c^{hi} dendritic cells. CD11c^{int} cells that also carry the marker B220 (pDCs) were however found in the vicinity of infected cells (Figure 3.12). These markers are characteristic for plasmacytoid dendritic cells which are known to be one of the major sources of type I interferons during infection (Asselin-Paturel et al. 2001). Although from the data presented here it is clear that *Listeria* infection does not trigger those cells to produce type I interferons (Figure 3.5).

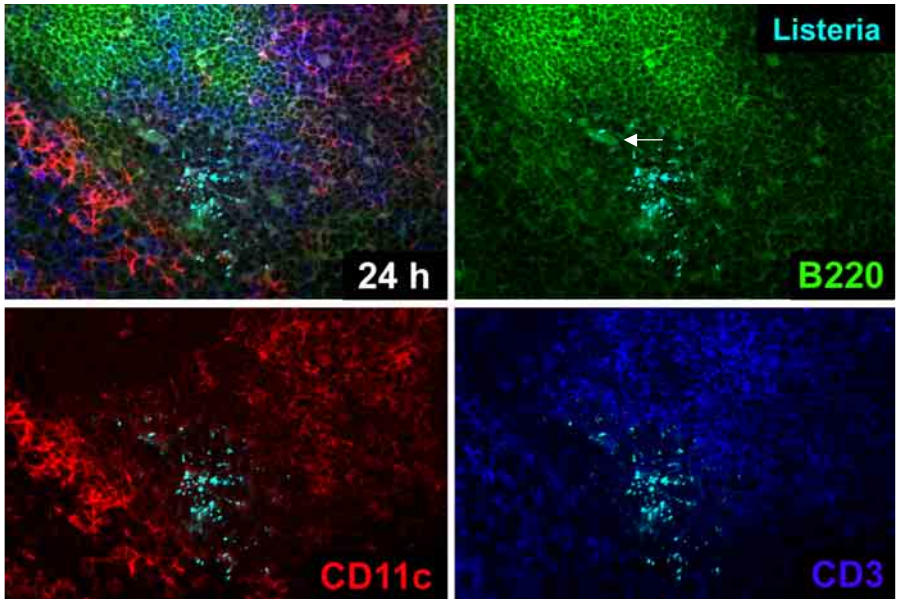


Figure 3.12 B220^{+} cells of DC phenotype in close vicinity of bacteria.

3.2.2 Low dose infection with *L. monocytogenes*

Since the above data were generated using very high dose, which might not reflect the scenario usually found, experiments using a sublethal infection dose were conducted to confirm the cytokine and chemokine induction patterns. To examine this, BALB/c mice were infected iv with *L. monocytogenes*: 2x10^{3} CFU. A longer time period was chosen for monitoring the infection, since the load of bacteria during such infections is comparatively low and the changes appear to be slowlier than during high dose infection. After 1, 2, 3 and 4 days PI mice were sacrificed, spleens removed and analyzed.

3.2.2.1 Estimation of *Listeria* load in the spleen of infected mice

At indicated time points after infection of mice with *Listeria*, spleens were removed, parts of them homogenized in 0.2% NP-40 in PBS, serial dilutions were plated on the BHI agar plates (Figure 3.13). The CFU of bacteria were increasing in infected spleen until the third day PI. Decrease of the bacterial numbers after this day is most likely due to the initiation of the specific immune response at this time. Thus the infection followed the expected time course.

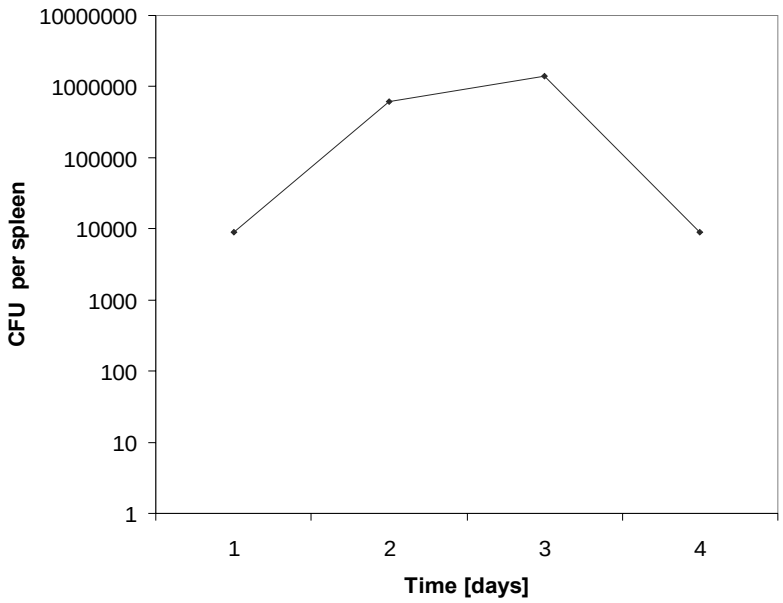


Figure 3.13 Growth curve of *L. monocytogenes* in the spleen of the iv infected BALB/c mice. Female mice were infected intravenously with $2x10^3$ (a low dose infection) CFU of *L. monocytogenes.* Number of injected *Listeria* was confirmed by plating aliquot of used inoculum on BHI plates. After 0, 1, 2, 3 and 4 days spleens were removed, parts of the organs homogenized in PBS supplemented with 0.2% NP-40 and plated on BHI agar. Load of bacteria per spleen after indicated time points was established after overnight incubation of plates at 37^oC. Growth curve was established as a mean of three mice per condition, $P<0.05$ were considered as significant.

3.2.2.2 Regulation of cytokine genes expression in spleen cell populations after a low dose *Listeria* infection

Spleens were removed from infected animals, adherent and total spleen cells prepared and RNA isolated. After reverse transcription induction of cytokine genes, normally regulated in activated macrophages, was analyzed using RT-PCR. However, the expression of some genes was too low to be detected on the agarose gel after standard RT-PCR amplification.

Increasing number of cycles lead to accumulation of unspecific products (data not shown). Such genes were for instance IL-6, iNOS, IFN non α4 and IFNβ (Figure 3.16). Since the course of induction of these cytokines during standard *Listeria* infection is well established and the major focus of this work concerned chemokines, attempt was made to improve the obtained data considering expression of chemokines.

Chemokines observed during a high dose infection were also detected under low dose conditions. The majority of them were exclusively expressed in adherent cell population. CCL7 and CCL12 were upregulated late; 4 days PI although in case of the latter chemokine a band was barely visible on the gel. CCL2, CCL3 and CXCL2 genes were upregulated 2 days PI and were increasing until day 4. The earliest upregulated chemokine gene turned out to be CXCL10. Already at 24 h PI a signal was obtained. The level of CCL5 remained constant during the course of infection, similarly to the infection with a high dose of *Listeria*.

CCL2 was the dominating chemokine early during a high dose infection. Therefore its early regulation was studied in many details during low dose infection. Since no signal was obtained by standard RT-PCR, a semi-nested RT-PCR was employed. With this improved sensitivity it was possible to demonstrate that expression pattern of the CCL2 gene was identical with the pattern obtained during a high dose infection. At 4 h PI CCL2 was upregulated and at later time points decreased almost to basic levels (Figure 3.15).

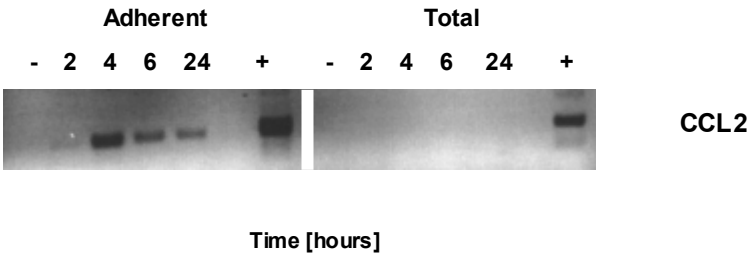


Figure 3.15 Semi-nested RT-PCR for CCL2. BALB/c mice were infected with a low dose of *L. monocytogenes*, RNA isolated after 0, 2, 4, 6 and 24 h PI, and semi-nested PCR employed to visualized the chemokine regulation. Obtained pattern is consistent with this observed in the case of a high dose of *L. monocytogenes*.

As expected, signals induced by the low dose of *L. monocytogenes* were much weaker compared to signals obtained with a high dose and the activation required longer time spans. Nevertheless, the gross sequence of events follow the sequence observed during a high dose conditions.

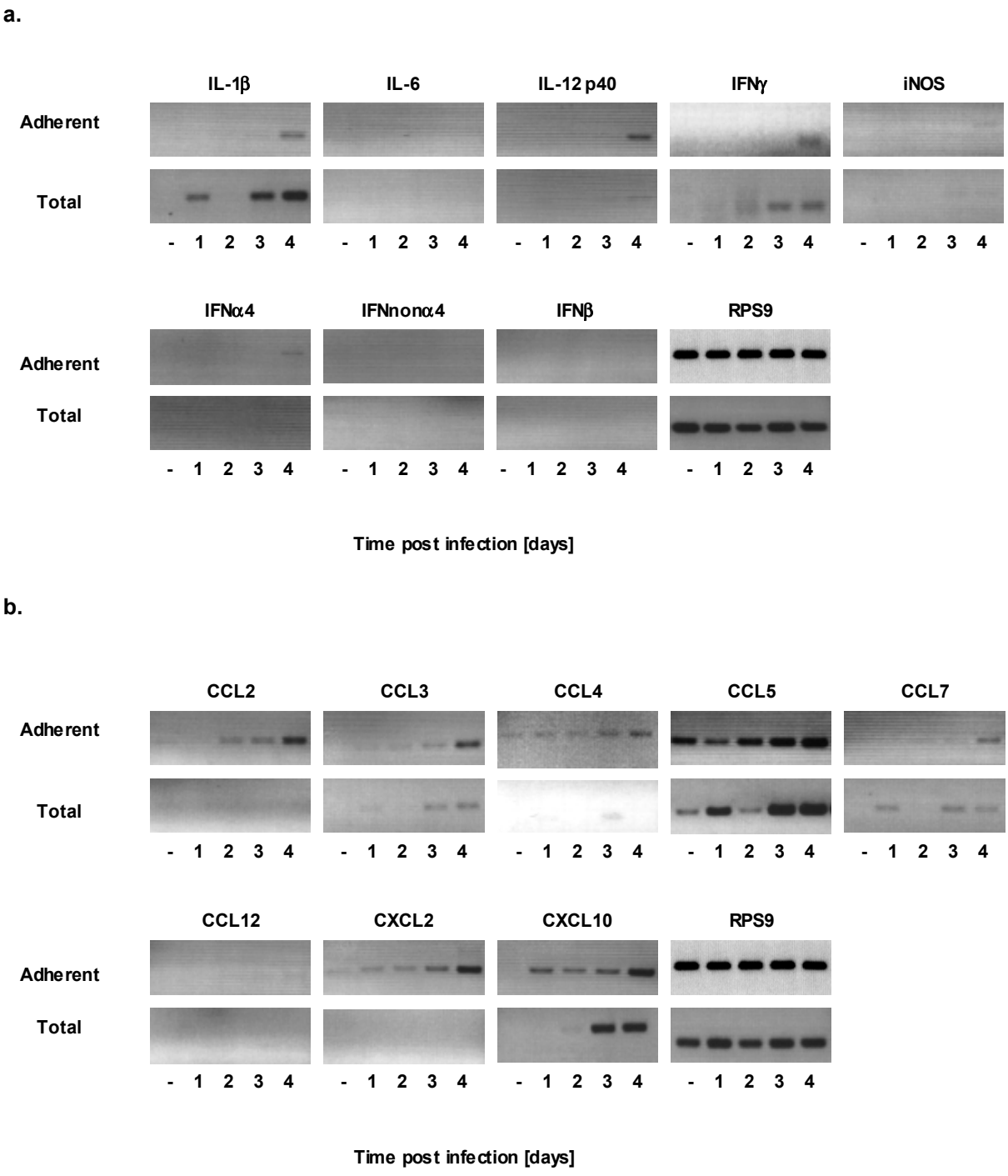


Figure 3.16 Regulation of cytokine and chemokine genes in spleen cells of mice infected with a low dose of *L. monocytogenes*. Mice infected intravenously with $2x10^3$ of *L. monocytogenes* were sacrificed at the indicated time points and spleens collected. Total and adherent cells from such spleens were analyzed for the specified mRNA by RT-PCR. The house keeping gene RPS9 was used as a positive control and for standardization.

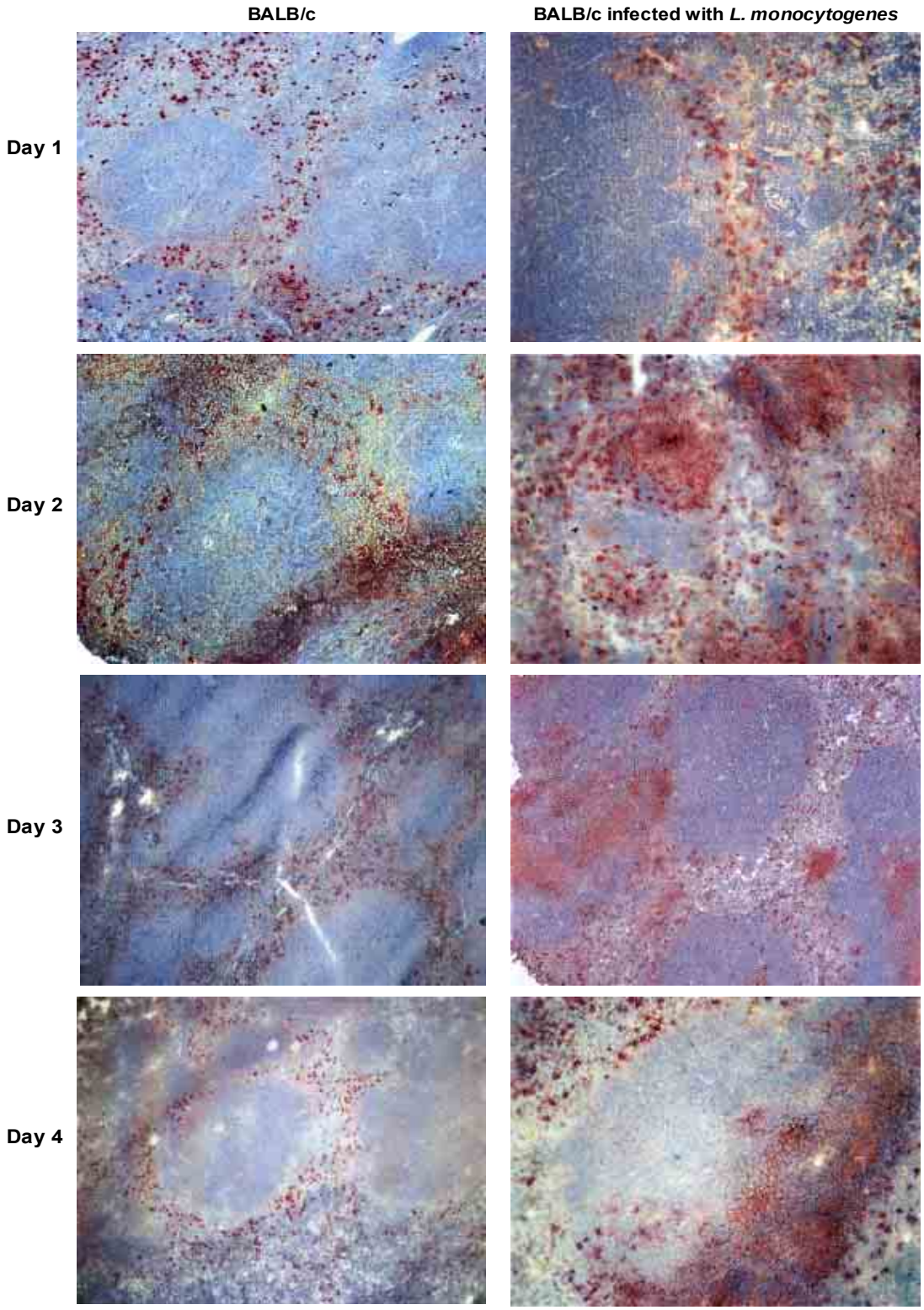


Figure 3.17 Architecture of the spleen of BALB/c mice after infection with *L. monocytogenes*. 1/3 of spleens from infected animals were snap-frozen in liquid nitrogen, cut into sections of 7µm and fixed in acetone (2 min at –20°C). After thawing and rehydrating, slides were stained as described above and analyzed using the optical microscope. Bacteria are stained in brown (PO+DAB) and Mac-1^+ cells in red (AP+Fast Red). For comparison sections of the uninfected spleen were also stained. Migration of the Mac-1^+ cells becomes obvious after 2 days PI, when these cells start clustering forming huge assembles containing *Listeria*.

3.2.2.3 Remodeling of the spleen architecture after a low dose infection of *Listeria*

Histology under low dose infection conditions was also carried out to see whether the delayed expression of chemokines was parallel to a delayed migration of phagocytes within the spleen. Anti Mac-1 staining of splenic crysections was therefore employed, since it would cover most of the cells affected.

Cell migration in the infected spleen is apparent at day 2 PI. Mac-1^+ cells, under normal conditions scattered in the whole red pulp begin to move, forming clusters between day 2-3 PI (Figure 3.17). At day 1 PI it is already possible to see single *Listeria* localized in the marginal zone (data not shown) and at later time points bacteria can be found exclusively inside Mac-1^+ macrophage clusters (Figure 3.18) and associated with Mac-1^+ cells.

A similar clustering of Mac-1^+ cells was already observed when a high dose of bacteria was applied iv, although the migration was much faster under these conditions and started at 4 h PI.

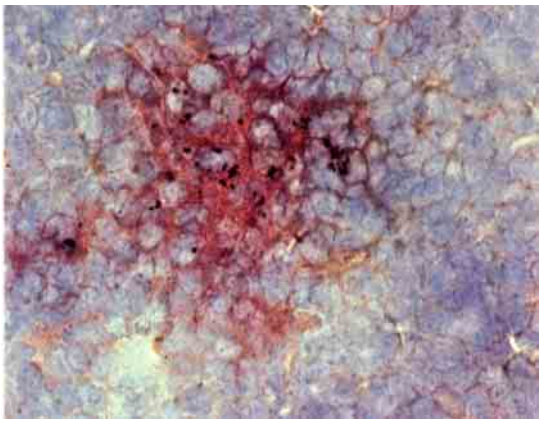


Figure 3.18 *Listeria* surrounded by Mac-1^+ cells. Spleens were isolated and prepared as described above. Migration of Mac-1^+ cells results in formation of huge clusters around the infected with *Listeria* cells.

3.2.2.4 Kinetics of changes in spleen cell composition after *L. monocytogenes* infection

Histology provides data only on the location of particular cells but does not allow the determination of quantitative changes in the composition of spleen cells. Therefore, to monitor the composition of spleen cells during infection, spleens were isolated at appropriate

time points PI and single spleen cell suspensions were stained for surface markers characteristic for the main cell populations of this organ. Data are summarized in Figure 3.19.

As expected a characteristic decrease of CD3 T cells occurs at the second day PI. This is most likely due to dying of unspecific T cells by apoptosis (Jiang et al. 2003). In contrast, macrophage populations are increasing. Especially at early time points one can observe an increase of the marginal zone macrophages ERTR9$^+$ and MOMA$^+$, while there is a drop at day 3 and 4. These cells have the earliest contact with the pathogen and might be at later time points the targets of specific CD8$^+$ T cells (Aichele et al. 2003). F4/80$^+$ macrophages of the red pulp increase only slightly during the course of infection, which is consistent with the statement, that they play minor role in anti-listerial defense. Finally, the cell population of Mac-1$^+$ macrophages that also contains a population of DCs increases late during infection. Their influx into the spleen is probably the result of the chemokines production by primarily infected macrophages and the upregulation of this molecule on resident splenic macrophages. Total DCs (CD11c^{hi}) and the subpopulation of the plasmacytoid DCs (CD11c^{int} B220$^+$), known to be the producers of IFNα/β, were also assessed. Plasmacytoid DCs (pDCs) start to increase by day 2 and remain at the same level while total DCs increase but show a drop at day 4 and increase at day 5 again. This might be due to cytolytic activity of T cells specific for listerial products that kill DCs at that time point. Neutrophils stained for Gr-1 show a more or less constant increase, while NK cells stained by anti DX5 fluctuated i.e. first increased then dropped at day 4 to strongly increase again. B cells numbers seemed not to be influenced very much, which is also in agreement with their minor role in anti-*Listeria* immunity.

Thus, very dynamic changes in spleen cell composition are observed during *Listeria* infection. These changes occur in parallel to the rearrangement of the architecture of the spleen. ERTR-9$^+$, MOMA-1$^+$ cells and pDCs seem to be the first cells influenced by bacteria. This is in agreement with the data obtained using a high dose of *Listeria*, even though it remains unclear how MOMA-1$^+$ macrophages are stimulated without direct contact with bacteria.

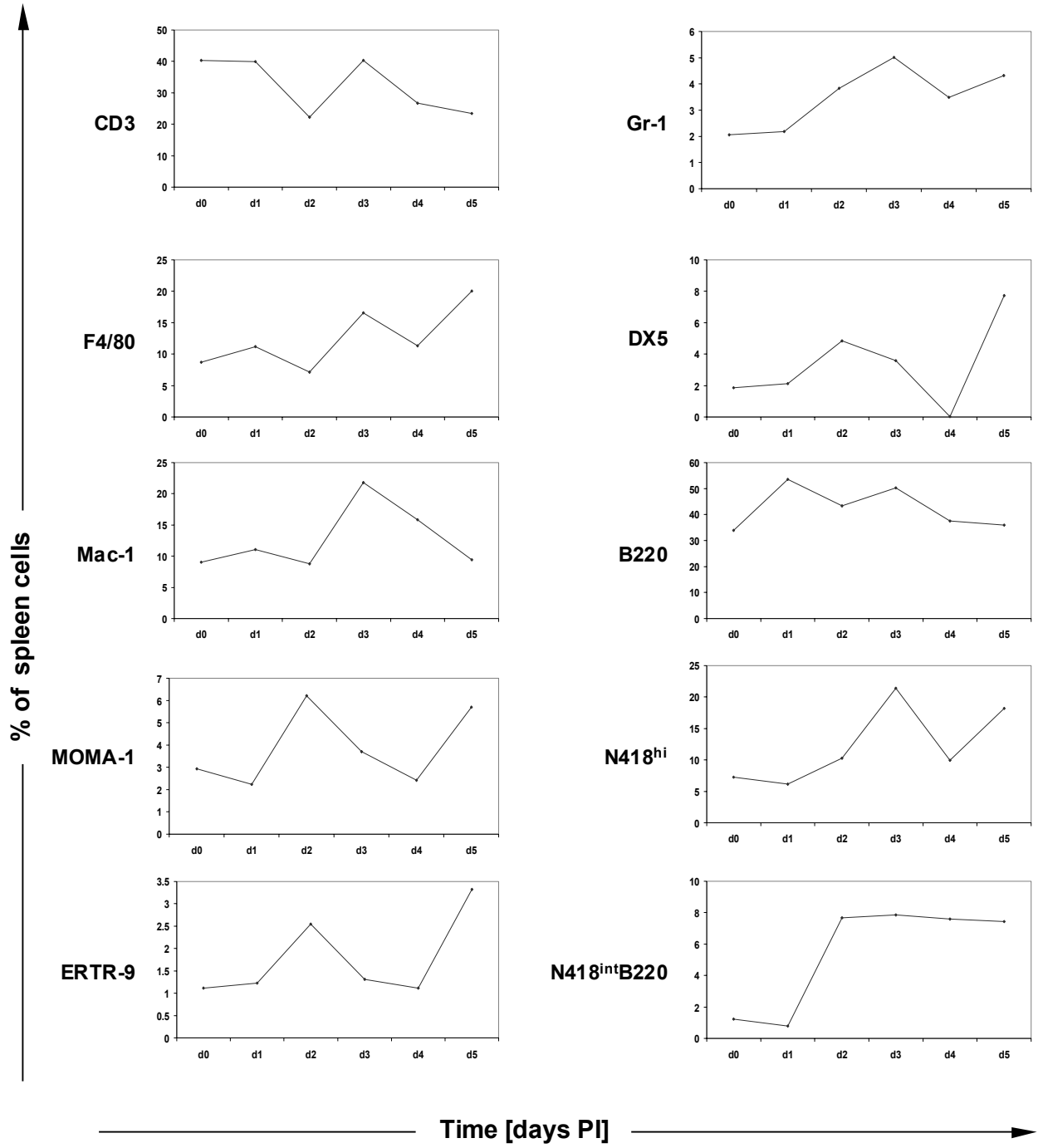


Figure 3.19 Changes in spleen cell populations after *L. monocytogenes* infection. Single cell suspensions were prepared from the spleen of infected animals. Cells were stained using Abs recognizing surface markers characteristic for the different spleen cell populations. Biotinylated Abs were revealed using SA-APC (Pharmingen, San Diego, CA), for 10 min on ice. After subsequent washing and staining of the dead cells with propidium iodide (Sigma-Aldrich, Deisenhofen, Germany) alive cells were analyzed by FACSCalibur.

3.3 Regulation of gene expression by different types of mice

Since heterogeneity of macrophages depends not only upon activation and maturation status but also on the genetic background (Mills et al. 2000), mice of other strains were examined in the early reactions during *L. monocytogenes* infection. It was expected that the difference in susceptibility of such mice should also become visible in the pattern of elicited reactivities.

3.3.1 The course of listerial infection in C57Bl/6 mice after a high dose iv infection

Resistant C57Bl/6 mice belong to the prototype strains with inflammatory responses upon infection directed into the Th1 pathway (Su et al. 2001). Macrophages from C57Bl/6 produce preferentially Th1 cytokines, have relatively abundant scavenger receptors on the surface and show high phagocytic activity, while BALB/c macrophages are directed more towards Th2 pathway, have relatively few scavenger receptors and their phagocytic activity is low (Su et al. 2001).

To investigate the reactions of C57Bl/6 mice upon *Listeria* infection and compare them to the results obtained for BALB/c, animals were infected iv with the high dose of bacteria – $5x10^5$. After 0, 2, 4, 6 and 24 h PI mice were sacrificed, spleens removed and spleen cell populations analyzed.

3.3.1.1 CFU of *Listeria* in the infected spleen

The *Listeria* load in the spleens was measured by plating of serial dilutions of the homogenized organs onto BHI agar plates and counting the CFU after overnight incubation at 37^{o}C. Since these mice are much less susceptible as BALB/c for *Listeria* infection, lower bacterial load in the spleen 24 h PI of such mice was not surprising (Figure 3.20). Mice did look healthier compared to BALB/c at this time point.

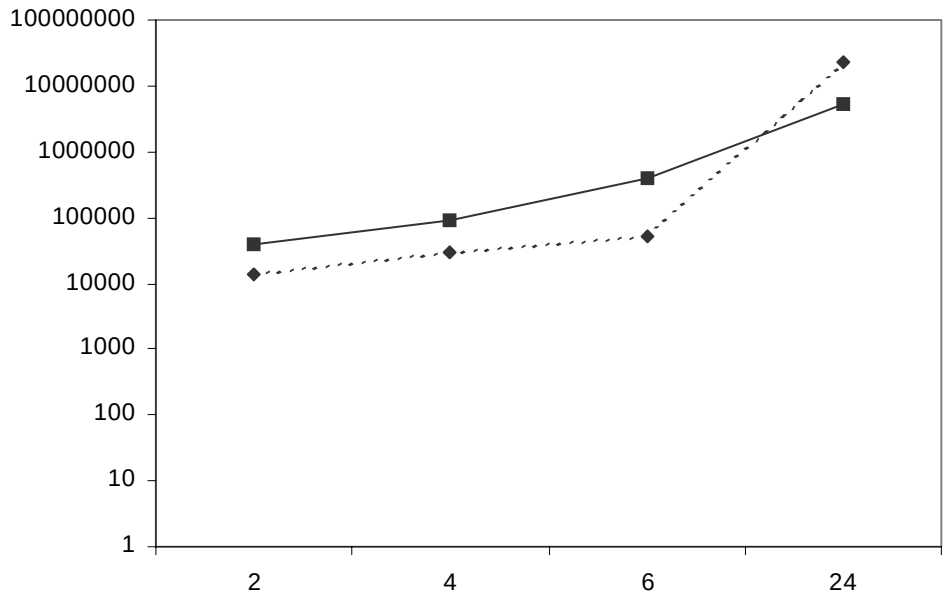


Figure 3.20 Evaluation of the number of *L. monocytogenes* in the spleen of C57Bl/6 and DBA/2 mice. Mice were infected intravenously with $5x10^5$ (a high dose infection) CFU of *L. monocytogenes.* Number of injected bacteria was confirmed by plating aliquot of used inoculum on BHI plates. After 0, 2, 4, 6 and 24 hours spleens were removed, parts of the organs homogenized in PBS supplemented with 0.2% NP-40 and plated on BHI agar. Load of bacteria per spleen after indicated time points was established after overnight incubation of plates at 37^oC. Growth curve was established as a mean of three mice per condition, $P<0.05$ were considered as significant.

3.3.1.2 Analysis of the gene expression pattern in the spleen of C57Bl/6 mice after infection with a high dose of *Listeria*

To compare the inflammatory response in C57Bl/6 mice with the established patterns of BALB/c, cDNA was prepared from the total and adherent spleen cell population and cytokine gene expression patterns were verified by RT-PCR amplification.

Similarly to BALB/c mice IL-1β and TNFα were highly upregulated during the whole course of infection. Expression of IL-6 was detectable after 24 h in adherent population, similar to BALB/c. Expression of IL-10 was regulated earlier, especially in adherent spleen cell population already after 2 h, reaching a peak at 4 h and decreased to the basic level after 24h (Figure 3.21). Surprisingly, IL-12p40 and iNOS were not regulated, or the produced levels were too low to detect using a standard RT-PCR. It is also possible that this dose was to low to induce this inflammatory cytokines at this early time points, since C57Bl/6 mice are much more resistant for *Listeria* infection than BALB/c. IFNγ production was restricted mainly to

the adherent spleen cells population, was early upregulated and increased until 24 h PI. This could be one of the factors responsible in this mice strain for anti-*Listeria* resistance. In contrast type I interferons were expressed mainly in the total population and not found in adherent, macrophage enriched population like in BALB/c.

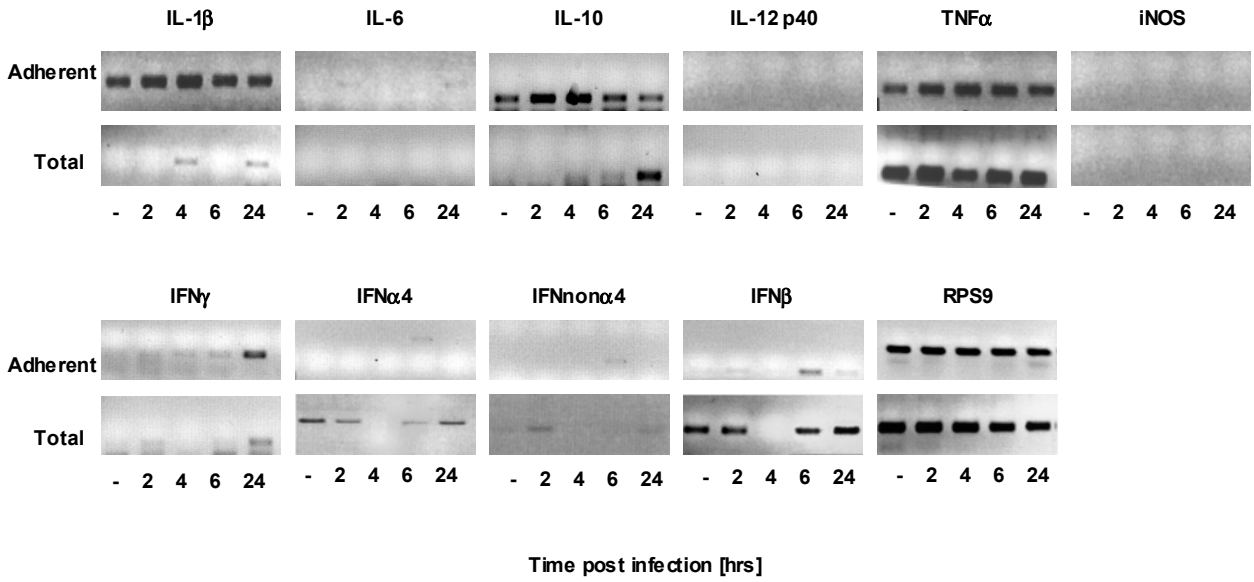


Figure 3.21 Cytokine gene expression after *Listeria* infection of C57Bl/6 mice.

Upregulation of chemokine genes was again mainly found in the adherent spleen cells population (data not shown). Interestingly, Real-Time RT-PCR reaction revealed a totally different chemokine gene regulation pattern compared to BALB/c mice (Figure 3.22). CCL2, the key player during the immediate response in BALB/c, in C57Bl/6 mice was hardly regulated after 4 h, and showed only a slight increase at 24 h. Since this chemokine is postulated to be a part of the Th2 response (Chensue et al. 1996;Karpus et al. 1997) its low upregulation in C57Bl/6 mice might be correlated with the resistance of this mouse strain to *Listeria*. Gene expression of CCL3, CCL4 and CXCL2 showed a similar pattern; after 4 h an initial increase of gene expression level, then a 2-3 times decrease after 6 h and at 24 h PI time point renewed high upregulation. CXCL10 increased already at 4 h PI, remained at the same level after 6 h and increased to high levels at 24 h. It is possible that such a high early genes expression upregulation leads to more efficient clearance of bacteria in this mice strain, since all these regulated chemokines are important for attraction and activation of monocytes, neutrophils, NK cells and T cells.

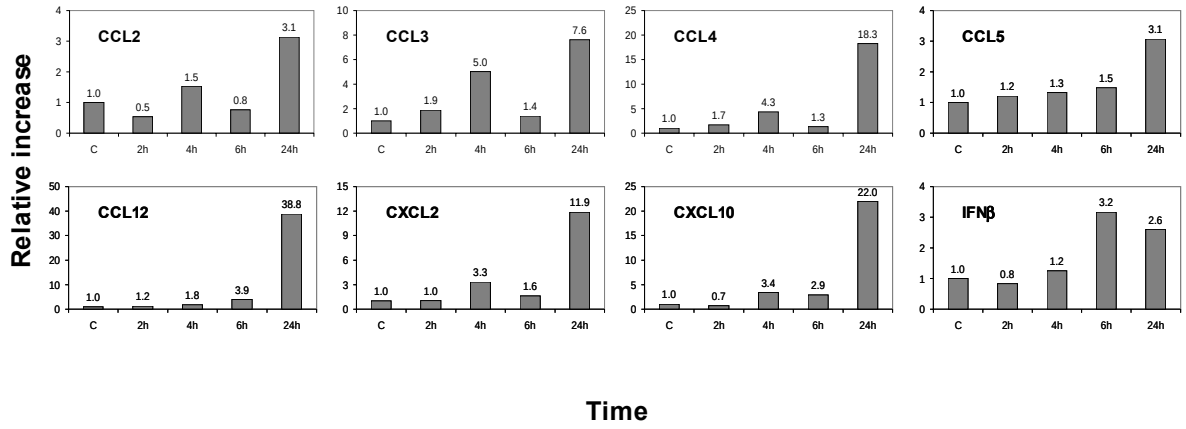


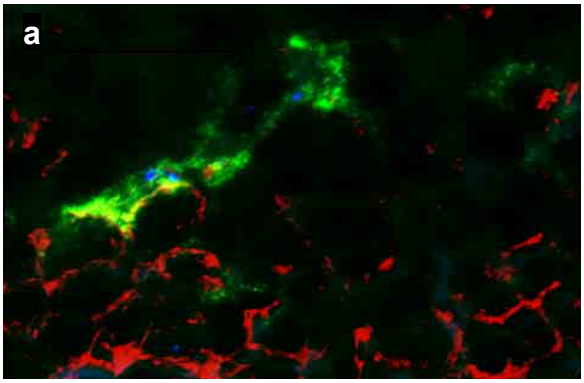
Figure 3.22 Chemokine genes expression pattern after C57Bl/6 mice infection with *L. monocytogenes*. CCL7 is not included since RT-PCR did not show any regulation of this chemokine gene at adherent spleen cell population and the estimation of the cDNA amount needed for a Real-Time RT-PCR was not possible in this case.

3.3.1.3 Spleen architecture of the C57Bl/6 mice after infection with *L. monocytogenes*

To follow changes in the spleen after *L. monocytogenes* infection spleens from infected C57Bl/6 mice were removed, cryosections prepared and staining of the main spleen cell populations carried out. Opposite to the BALB/c mice Mac-1^+ cells formed only small clusters after relatively long time PI (data not shown).

Similarly to the infected BALB/c mice bacteria were found at 6 h PI exclusively in ERTR-9^+ macrophages (Figure 3.23). At 24 h ERTR-9^+ macrophages associated with *Listeria* were found in small clusters with Mac-1^+ cells (Figure 3.23).

It is possible that significantly lower upregulation of the chemokine genes in infected C57Bl/6 mice is responsible for this late macrophage infiltration and formation of the relatively small cell clusters.



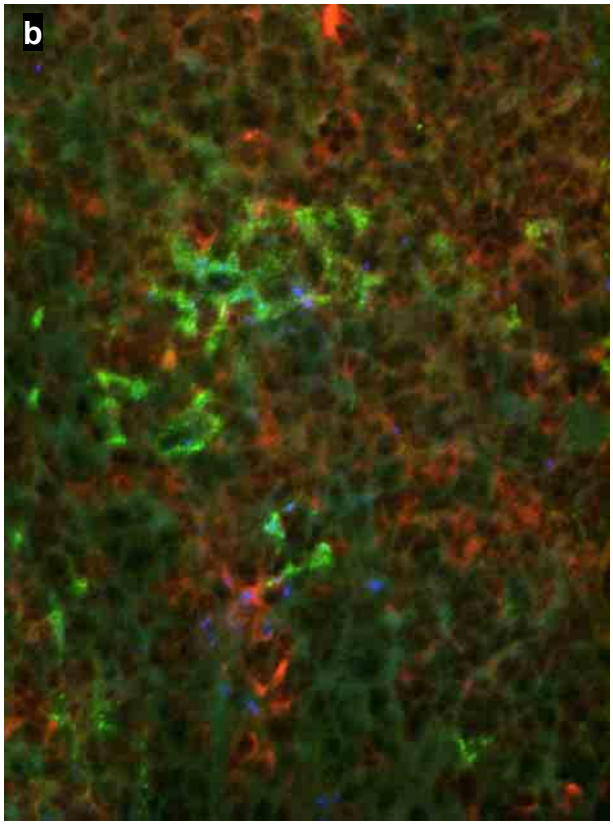


Figure 3.23 Late clustering of Mac-1$^+$ and ERTR-9$^+$ cells in response to *Listeria* infection. Cryosections of the infected spleens from C57Bl/6 mice were stained with Abs against **Mac-1**, **ERTR-9** and ***Listeria***. Number of bacteria in the spleen of C57Bl/6 mice was distinctly lower than in infected BALB/c mice. At 6 h PI *Listeria* are colocalized exclusively with ERTR-9$^+$ macrophages (**a**). Infiltration of the Mac-1$^+$ cells was not visible earlier then 24 h PI and clusters formed at this time around infection foci are relatively small in comparison to BALB/c mice. Inside the clusters ERTR-9$^+$ macrophages colocalized with *Listeria* could also be found (**b**).

3.3.2 *L. monocytogenes* infection of DBA/2 mice

Next DBA/2 mice were tested for an anti-*Listeria* response. These mice are highly susceptible to *L. monocytogenes* infection, but the exact mode of the inflammatory response, i.e. Th1 or Th2 is not established in this strain. DBA/2 mice are depleted in the C5 component of complement. Therefore, the absent complement activation and the lack of the anaphylatoxin fragment C5a might contribute to the susceptibility and the overall reactions.

To be consistent with previous experiments, DBA/2 mice were infected iv with a high dose of *L. monocytogenes* ($5x10^5$), after 0, 2, 4, 6 and 24 hours spleens were removed and analyzed.

3.3.2.1 Bacteria load in infected spleen of DBA2 mice

Fragments of spleens were homogenized, plated in serial dilutions on BHI agar and after overnight incubation at 37^{o}C CFU per spleen was estimated. The susceptibility of these mice is obviously higher then that of C57Bl/6 mice (Figure 3.20). The bacterial burden in the spleen increases first slowly between 2 and 6 h PI and then grows to levels higher than in BALB/c mice. At the 24 h PI time point mice appear severely affected.

3.3.2.2 Cytokine and chemokine expression pattern in DBA/2 mice after a high dose of infection with *Listeria*.

The expression pattern of inflammatory cytokines and chemokines in the spleens of infected DBA/2 animals was investigated. Thus RNA was isolated from total and adherent spleen cell population at time points mentioned above and RT-PCR was employed to amplify the cDNA prepared from these samples. IL-1β and TNFα were expressed like in BALB/c (Figure 3.24). IL-6 was coming up late, after 24 h and the signal was stronger than that in BALB/c. IL-10 as well as IL-12 was earlier upregulated then in the two strains tested before. IFNγ was induced by 4 h and increased until 24 h PI. Interestingly, type I interferons expression was found in the adherent population, which was not observed in the two other strains. The increased intensity of the signals might be partly due to the higher bacterial load at 24 h. On the other hand in DBA/2 particular cell types might respond differently e.g. in this mouse strain pDCs might be induced to produce type I interferons.

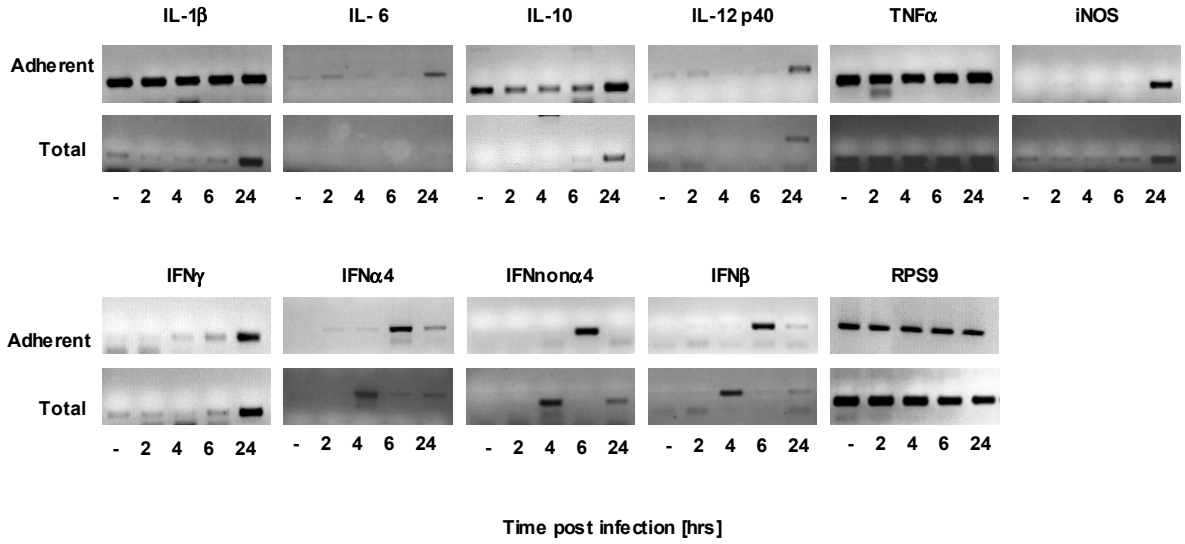


Figure 3.24 Cytokine gene expression after *Listeria* infection of DBA/2 mice. Mice were infected iv, spleen removed at indicated time points, RNA from the spleen cells isolated and after RT cDNA analyzed in RT-PCR.

To estimate the expression level of inflammatory chemokine genes in adherent, macrophage enriched spleen cell population from infected DBA/2 mice, Real-Time RT-PCR was carried out. The pattern of expression differed from the patterns of the two other mice strains examined. As soon as 2 h PI expression of CCL12 and CXCL10 could be detected. Such an early regulation of these genes was not observed in other mice strains tested (Figure 3.25).

These genes increased until 24 h PI reaching very high levels. CCL7 was upregulated after 6h and then decreased significantly. At 24 h the majority of chemokines was upregulated, except for CCL5, which seems to be not regulated at all, similarly to mouse strains BALB/c and C57Bl/6. CCL4 could not be quantitated, since the total product obtained after RT-PCR amplification was not high enough to calculate a cDNA amount for Real-Time RT-PCR.

Little is known about the Th bias of DBA/2 mice upon infection. From the data obtained, one could conclude that the inflammatory responses in such mice are directed more into a Th1 pathway i.e. high production of CCL12 and CXCL10, known to be potent chemokines for Th1 pathway activation in addition to the early and strong upregulation of IFNγ in the adherent population. In agreement with this only low levels of IL-10 are induced in these mice.

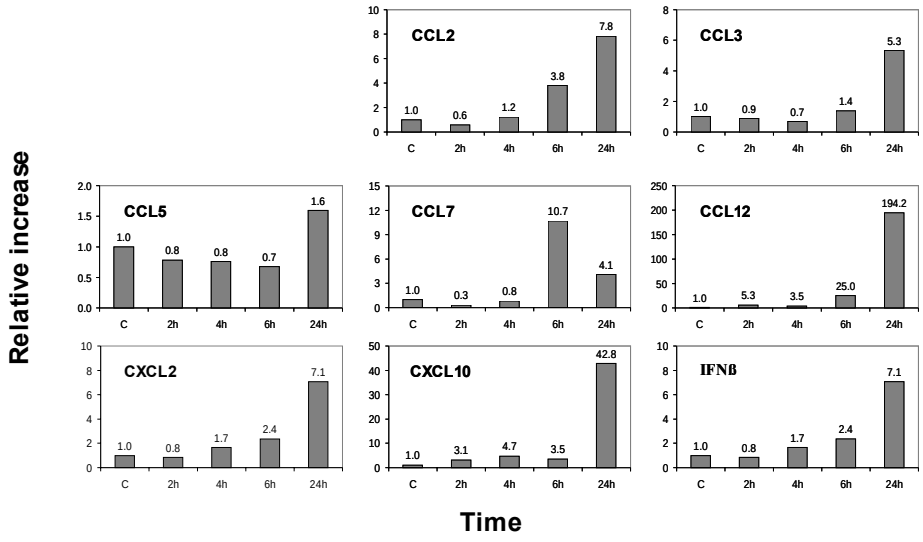


Figure 3.25 Quantitation of chemokine gene expression in adherent spleen cell population after *Listeria* infection of DBA/2 mice. CCL4 is not included since RT-PCR did not show any regulation of this chemokine gene at adherent spleen cell population and the estimation of the cDNA amount needed for a Real-Time RT-PCR was not possible in this case.

3.3.2.3 Changes in spleen architecture after infection of DBA/2 mice with *L. monocytogenes*

Differential regulation of the cytokines and chemokines in infected DBA/2 mice in comparison to the BALB/c and C57BL/6 mice should also lead to distinct changes in the spleen architecture. To investigate that, spleens from infected mice were frozen in liquid nitrogen, 7µm cryosections prepared and stained with specific antibodies. As expected, the most obvious outcome was clustering of Mac-1^+ cells during the course of infection.

However, in the spleens of infected DBA/2 mice relocation of these macrophages is delayed compared to BALB/c mice and similar to C57BL/6 mice (data not shown). In addition clusters formed at 24 h PI are much smaller then those found in BALB/c strain (Figure 3.26). Once again B cells seem not to play any role in anti-*Listeria* immunity, since the structure of follicles did not change during the course of experiment. $CD3^+$ T cells partially migrate out of the white pulp and could be observed in the clusters.

The comparatively small rearrangements in the spleens of infected DBA/2 mice might be due to the low and late upregulation of the cytokine and chemokine genes. CCL2, thought to play a key role during the initial phase of *Listeria* infection and to be responsible for the early clustering of macrophages in BALB/c mice is upregulated at relatively low levels and only at 24 h PI.

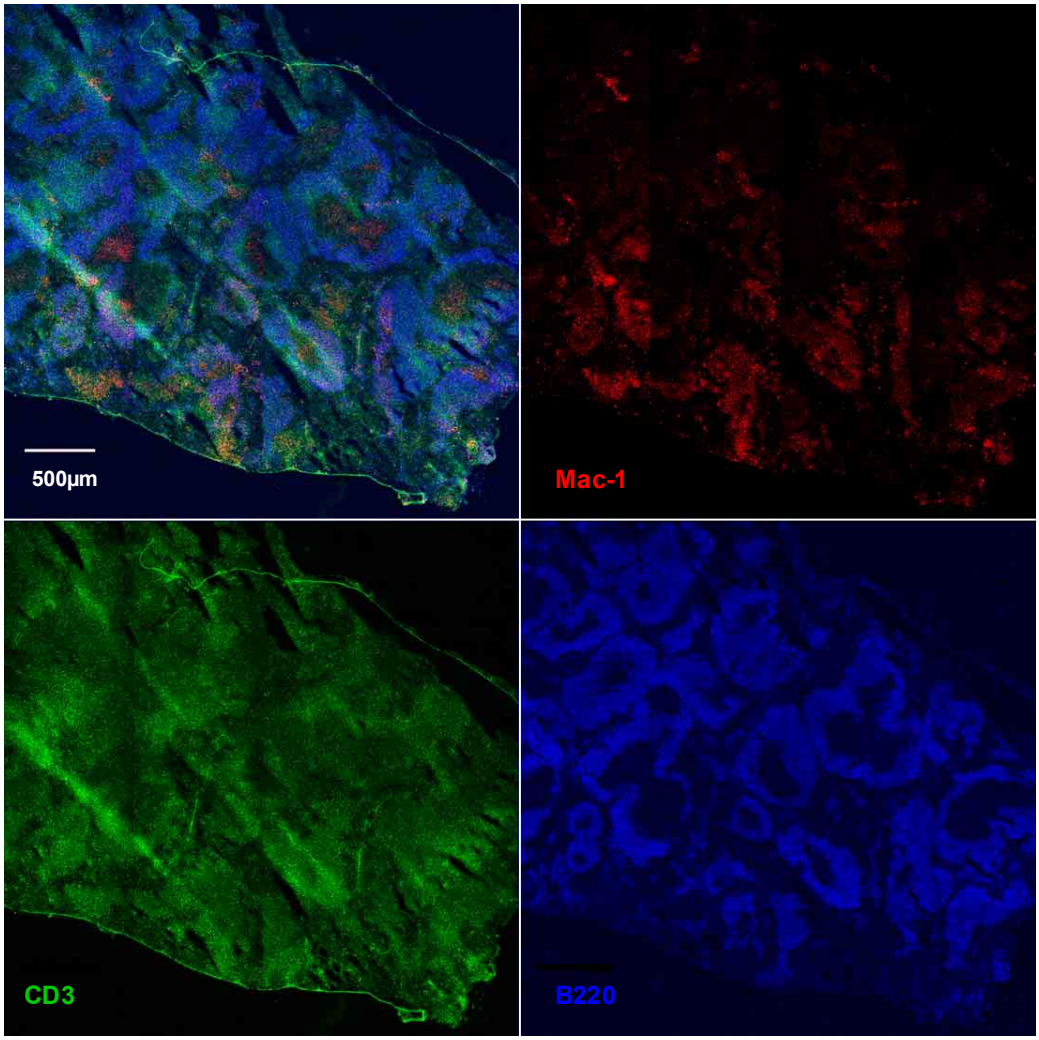


Figure 3.26 Weak clustering of the Mac-1^+ cells in the spleen of infected DBA2 mice at 24 h PI. Mice were infected with a high dose of *L. monocytogenes* ($5x10^5$), spleens removed at appropriate time points and frozen. 7µm cryosections were made and stained as described above. Mac-1^+ cells are stained in red, **CD3** in green and **B220** in blue.

3.4 Impact of L. monocytogenes infection on the cytokine and chemokine gene expression pattern by different splenic macrophage and DC populations

Adherent cells, although strongly enriched in macrophages can only give an approximation of the genes regulated in different cell types during the initial phase of *Listeria* infection. They still represent a heterogeneous population consisting of all macrophages and DCs. Thus the contribution of a particular cell population cannot be assessed. For instance the ERTR-9$^+$ cells appear the only cell to be infected. Hence, their contribution to the expression of inflammatory chemokines was of particular interest, since they might be the initiators of the reactions that follow. In addition, the dynamics that might be occurring under these circumstances i.e. changes in the composition of these cells cannot be accounted for. Therefore, spleen cells from infected BALB/c mice were isolated, depleted of T and B cells, populations of interest sorted and cDNA prepared. Expression of chemokine genes established by RT-PCR was compared between these populations.

3.4.1 Cell sorting of the main macrophage and DC populations from the spleen of infected BALB/c mice

BALB/c were infected with a high dose of bacteria ($5x10^5$) and after T and B cells depletion ERTR-9$^+$ macrophages were isolated from infected animals at 4 h and 24 h PI using a FACS sorter DiVa. For comparison different cells also found in the adherent spleen cell populations – MOMA-1$^+$, F4/80$^+$, CD11c^{hi}B220$^-$ and CD11c^{int}B220$^+$ were sorted and tested for cytokine and chemokine expression. Sorting of these populations, especially ERTR-9$^+$ and MOMA-1$^+$ was difficult because of the low number of such cells and low surface expression of these markers. Nevertheless, reanalysis of these sorted populations revealed a purity of 85-90% of these cells (Figure 3.27).

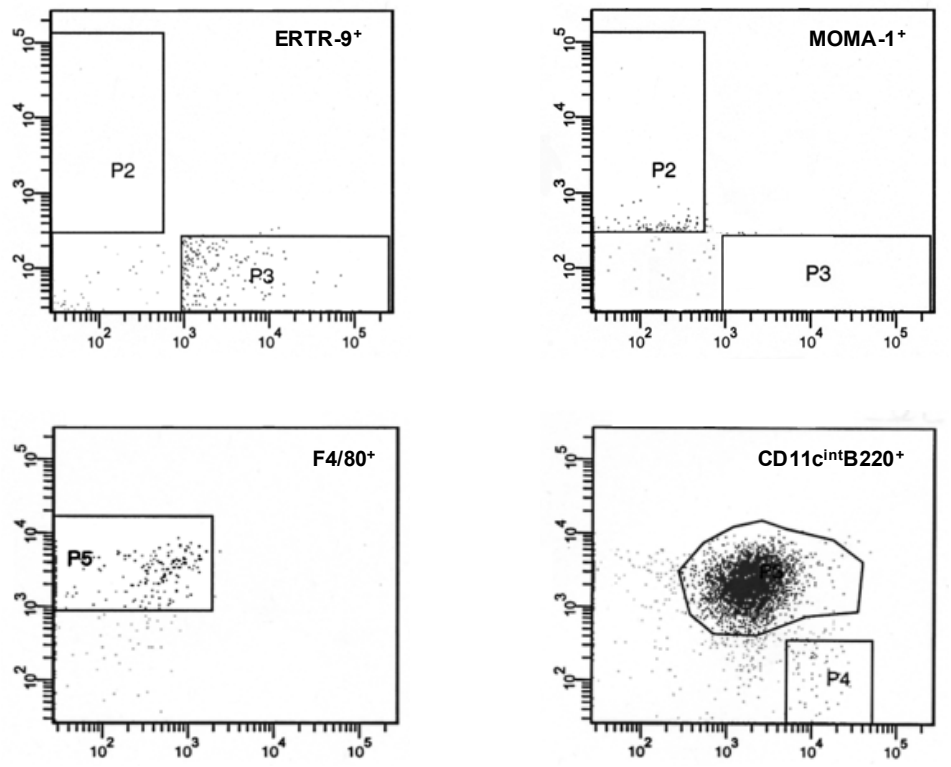


Figure 3.27 Reanalysis of the sorted spleen cell populations from *L. monocytogenes* infected BALB/c mice. Mice were infected with $5x10^5$ CFU of *L. monocytogenes*, after 0, 4 and 24 h PI spleens removed, depleted in T and B cells and splenic macrophage and DC populations sorted out using a FACS Vantage DiVa (Becton Dickinson, Heidelberg, Germany). For every condition pools of 10 mice were used.

3.4.2 Cytokine and chemokine expression pattern in sorted populations of macrophages and DCs

First, the two types of marginal zone macrophages were analyzed, since these cells are the first ones to encounter the pathogen. RNA from sorted cells was isolated, cDNA prepared and expression of chemokine genes was investigated by RT-PCR. RT-PCR revealed that during the initial phase of *Listeria* infection ERTR-9^+ population is the main source of CCL2 as expected (Figure 3.28). Thus, the observed high increase of this chemokine at 4 h PI in the adherent spleen cell population is most likely due to these cells. Another possible contributor

of this early expression are CD11c^{int}B220^{+} cells, although the level of CCL2 is extremely low in comparison to the ERTR-9^{+} cells (Figure 3.29). At 4 h PI ERTR-9^{+} cells are also the main producers of other chemokines like CXCL2.

MOMA-1^{+} macrophages seem to play a minimal role in the production of the inflammatory chemokines. This is consistent with the hypothesis that these cells have totally different function, since at an early stage of infection they migrate into the B cell follicle. The only chemokine highly expressed by them at 4 h PI is the CXCL2, known to be a functional homologue of human IL-8 that plays an important role in neutrophil activation and degranulation (Rollins 1997). F4/80^{+} macrophages seem not to play a major role early during *Listeria* infection, since no inflammatory chemokine was upregulated in this population. Surprisingly DCs, CD11^{hi} as well as CD11c^{int} B220^{+} population already at 4 h PI initiate an inflammatory chemokine response. Plasmacytoid DCs express CCL2 as mentioned above and both populations express CXCL10, a chemokine that attracts the Th1 and NK cells. At 24 h PI these two cell populations also produce IFNγ obviously contributing to the Th1 bias of the anti-*Listeria* response. At 24 h all the cell populations produce all chemokines.

Interestingly, none of these populations contribute to the type I interferons production, which is in agreement to earlier data indicating that the producers of these cytokines belong to the non adherent spleen cell population.

Since in this work CCL2 is considered as a key chemokine for the immediate immune response during the *Listeria* infection in BALB/c mice, expression of this chemokine in the marginal zone macrophages was analyzed using quantitative Real-Time RT-PCR. As demonstrated in Figure 3.31, ERTR-9^{+} cells are extremely potent producers of this chemokine already at the early stage of infection. It is not clear whether the initial production of CCL2 is intrinsically limited to this macrophage population, or whether it is due to the exclusive infection of these cells at the early stage of infection. Results of experiments obtained in this work suggest rather the second scenario since at later time points other cells also are able to produce this chemokine.

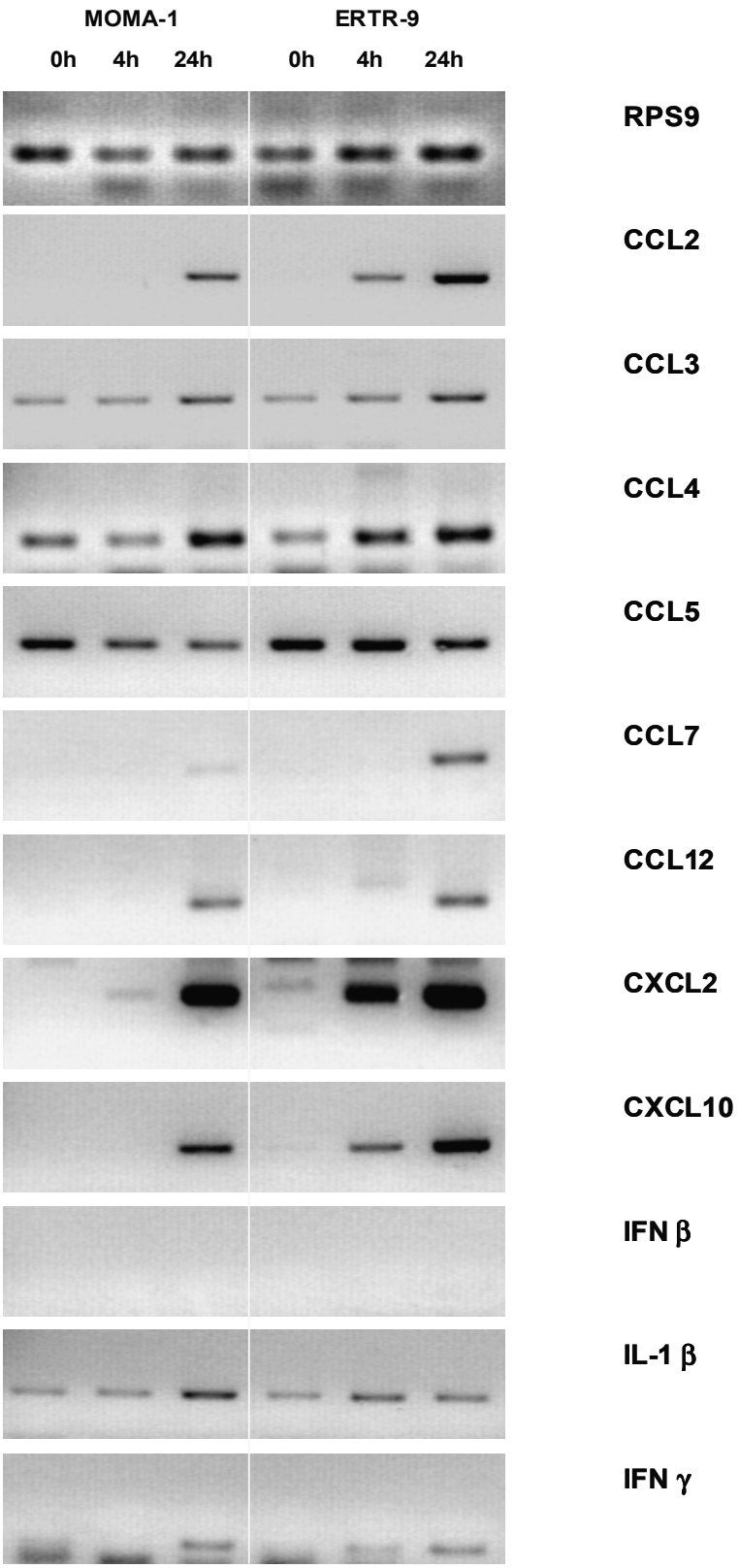


Figure 3.28 Regulation of cytokine and chemokine gene expression by marginal zone macrophages ERTR-9$^+$ and MOMA-1$^+$. Mice were infected with 5x10^5 CFU of *L. monocytogenes*, after 0, 4 and 24 h PI spleen removed, marginal zone macrophage populations sorted and RNA isolated. RT-PCR from prepared cDNA was employed to see gene expression pattern.

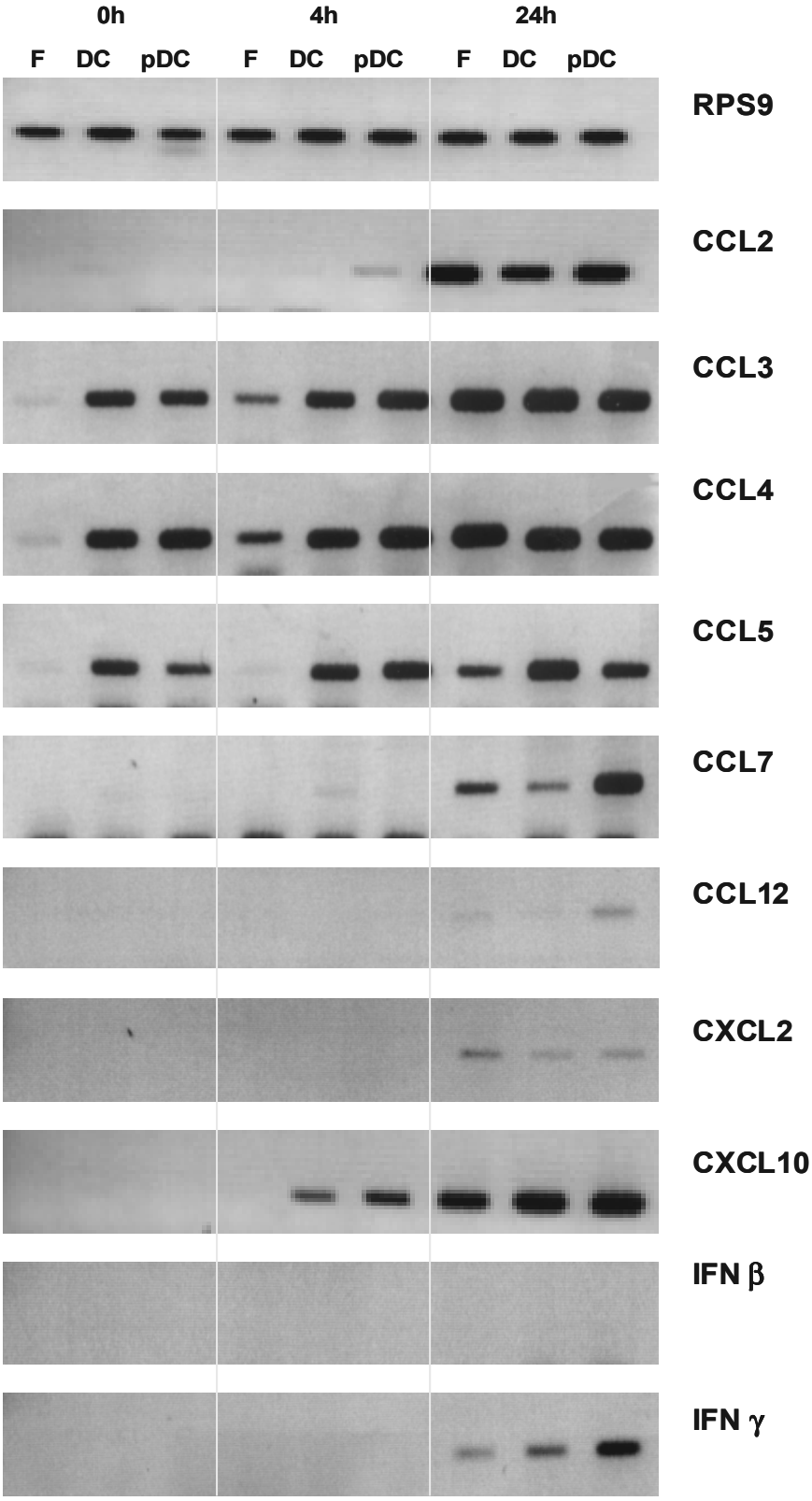


Figure 3.29 Regulation of cytokine and chemokine gene expression by red pulp macrophages F4/80^{+} (F), dendritic cells CD11c^{hi}B220^{-} (DCs) and CD11c^{int} B220^{+} (pDCs), isolated from infected BALB/c mice. Mice were infected with 5x10^{5} CFU of *L. monocytogenes*, after 0, 4 and 24 h PI spleen removed, macrophage and DC populations sorted and RNA isolated. RT-PCR from prepared cDNA was employed to see gene expression pattern.

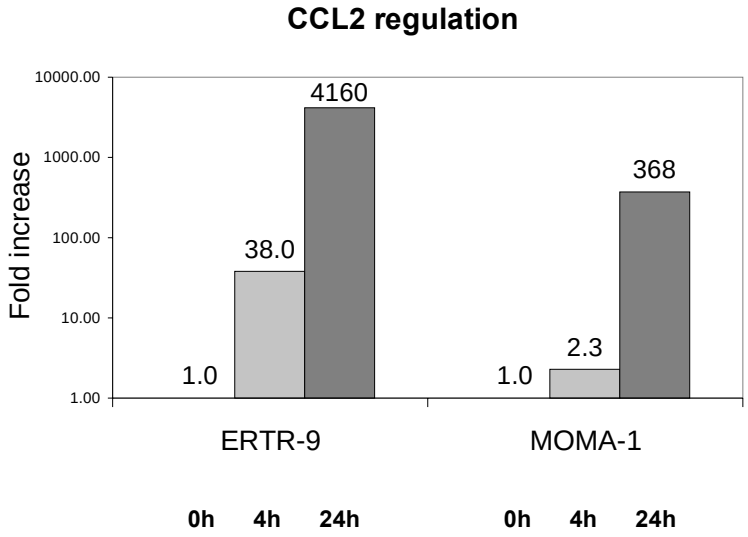


Figure 3.30 CCL2 regulation in ERTR-9$^+$ and MOMA-1$^+$

3.5 Blocking of the CCL2 function in infected with L. monocytogenes mice, using polyclonal anti-CCL2 antibodies

During these experiments it became clear that CCL2 is the chemokine playing a dominating role at the early stage of *Listeria* infection in BALB/c mice. To further elaborate on the importance of this chemokine the function of this protein should be blocked. There are several possible ways to achieve this. The receptor of CCL2 could be blocked by antibodies (Abs), an antagonist could be applied or the protein could be neutralized by a specific antibody. Blockade of CCR2 function raises the problem that not only CCL2 is an agonist for this receptor (Franci et al. 1995;Combadiere et al. 1995) (Sarafi et al. 1997). Thus, such treatment would also influence the activity of CCL7 and CCL12. Therefore direct inhibition of the function of the secreted chemokine with specific antibodies was chosen.

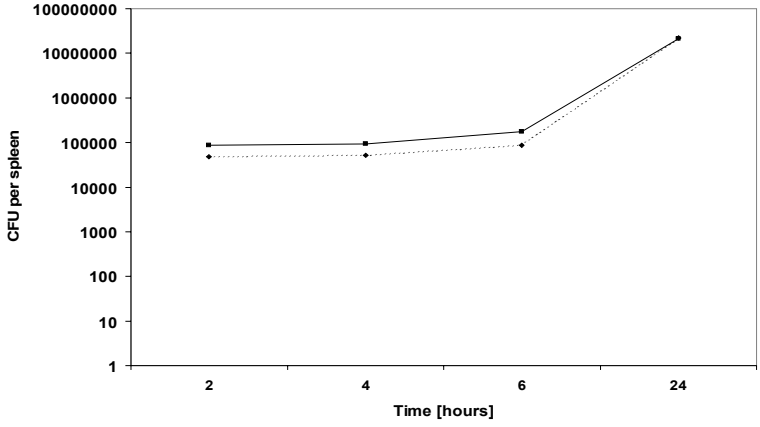
3.5.1 Infection of the CCL2 depleted BALB/c mice with a high dose of *L. monocytogenes*

Since most of the experiments were carried out with a high dose of *L. monocytogenes*, this dose was chosen for the experiments using animals with inhibited CCL2 function.

BALB/c mice were injected with 10µg/ml of anti-CCL2 antibodies 1 h before *Listeria* infection. Even lower amounts of antibodies (5µg/ml) are known to neutralize the *in vivo* activity of CCL2 (Lloyd et al. 1997). After 0, 2, 4, 6 and 24 h spleens were removed, CFU determined and RNA was isolated from the adherent cell population. Subsequent RT-PCR allowed analysis of the gene expression pattern. Results then were compared with the pattern obtained from untreated BALB/c mice infected in parallel.

3.5.1.1 Different *Listeria* number in the spleens of infected BALB/c mice with blocked CCL2 function, in comparison to the untreated animals

Fragments of spleens of infected mice were harvested at the indicated time points, homogenized and plated in serial dilutions at BHI agar plates. After overnight incubation in 37°C CFU were determined and growth curves compared between untreated and antibody treated animals. Two fold differences were consistently observed in growth curves between 2 and 6 h PI (Figure 3.31). Anti-CCL2 treated mice had a lower bacterial load in the spleen, but similarly to the untreated BALB/c mice were not able to control the infection, since at 24 h PI identical CFU were observed.



	2	4	6	24
BALB/c	86000	93000	175000	22000000
BALB/c + αCCL2	47400	51000	88000	21200000

Figure 3.31 Comparison of the growth curves of *Listeria* in the spleens of anti-CCL2 treated animals or untreated BALB/c mice after a high dose infection. Mice were infected with $5x10^5$ CFU of bacteria, after appropriate time points animals were sacrificed, spleens removed and aliquots plated on BHI agar plates after homogenization.

3.5.1.2 Differences in cytokine and chemokine production between BALB/c treated with anti-CCL2 antibodies or PBS, infected subsequently with a high dose of *Listeria*

The effect of the Abs treatment on CFU was not well pronounced; nevertheless expression pattern of cytokine in the adherent cell population was strongly influenced. Also spleen sizes differed between both groups – organ sizes from the Abs injected mice appeared smaller in comparison to the untreated controls.

RNA and cDNA were prepared from adherent cell population and RT-PCR employed to establish gene expression pattern in animals depleted in CCL2. Results were compared to untreated BALB/c mice infected in the same experiment. IL-1β was upregulated very early and remained at the high level throughout the whole experiment in both groups of mice as seen before (Figure 3.32). Expression of IL-10, IFNγ and CXCL10 increased much earlier in animals treated with anti-CCL2 Abs. Since in BALB/c animals IFNβ was not upregulated upon *Listeria* infection in adherent cell population it was surprising to see a weak upregulation in Ab treated mice at 24 h PI. CCL2, CCL4, CCL5, CCL7, CCL12 and CXCL2 were similarly regulated in both groups of mice, but in the group treated previously with Abs these chemokines were expressed at slightly higher levels. IFNγ and CXCL10, factors known to be responsible for Th1 pathway occur earlier in Ab treated mice. This might support a stronger Th1 inducing cytokine milieu and might be responsible for keeping the bacteria better in check at early time points.

3.5.2 Low dose *Listeria* infection of anti-CCL2 treated mice

Since it is postulated that CCL2 is essential only at the early stage of infection (Warmington et al. 1999;Traynor et al. 2002) and later its function can be replaced by other chemokines signaling via CCR2 receptor (CCL7 and CCL12), it was interesting to see if inhibition of the CLL2 function has any impact on the course of *L. monocytogenes* infection at the later time points. To investigate later time points it was necessary to use low dose of *Listeria* infection, because as shown before, mice treated with a high dose of bacteria are not able to control the infection after 24 h PI.

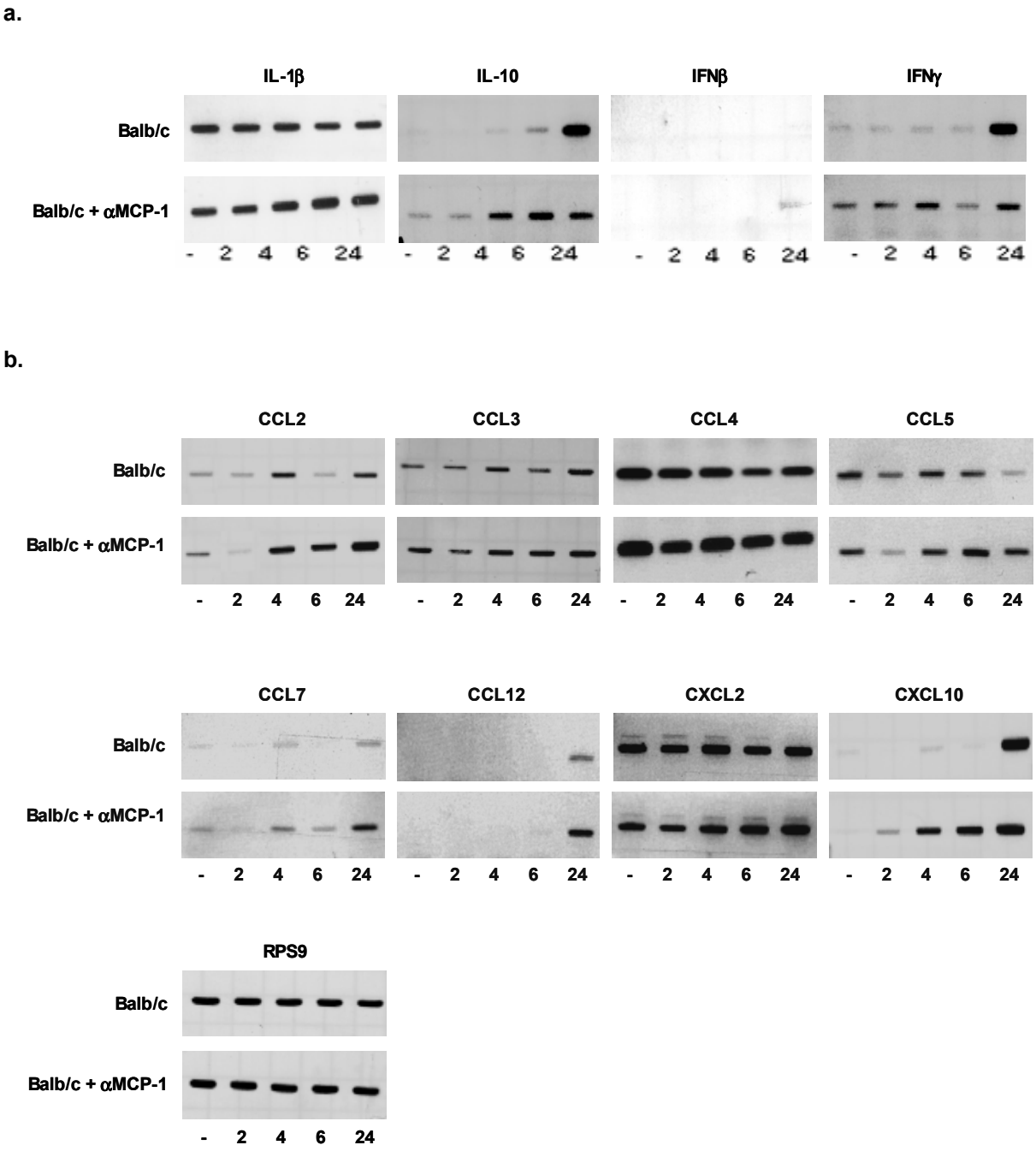
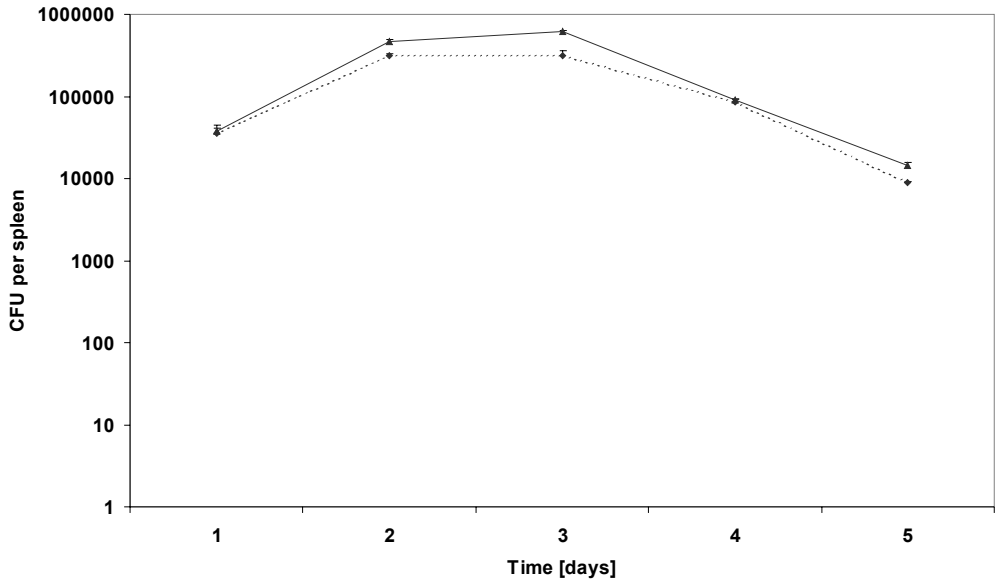


Figure 3.32 Comparison of cytokine and chemokine gene expression patterns between anti-CCL2 Abs treated and untreated BALB/c mice infected with a high dose of *L. monocytogenes*. BALB/c mice were injected with 10µg of anti-CCL2 antibodies 1 h before *Listeria* infection. As a control mice injected with PBS were used. Both groups were subsequently infected iv with $5x10^5$ CFU of *L. monocytogenes*. At appropriate time points animals were sacrificed, spleens removed and RNA isolated from the adherent spleen cells. To investigate gene expression patterns RT-PCR amplification of prepared cDNA was employed.

3.5.2.1 Evaluation of CFU of *L.monocytogenes* in the spleen of infected mice treated previously with anti-CCL2 antibodies

BALB/c mice treated with PBS (control mice) or anti-CCL2 antibodies were infected with $2x10^3$ CFU of *Listeria*, at 0, 1, 2, 3, 4 and 5 days PI spleens were removed, parts of them homogenized and serial dilutions plated on the BHI agar plates. After overnight incubation at 37^oC CFU were counted and growth curves established. Growth curves obtained from BALB/c control mice and mice treated with Abs were then compared.

Similar to the infection with a high dose, animals treated with antibodies against CCL2 showed a slightly lower load of bacteria in the spleen (Figure 3.33). Although the difference is small, it is consistent with the data obtained from a high dose of infection, it was reproduced in a second experiment and the CFUs were similar in all animals tested at one time point.



BALB/c	———	37671	472929	617854	90323	14589
BALB/c + αCCL2	.....	35393	315033	316136	85516	8896

Figure 3.33 Comparison of the growth curves of *L. monocytogenes* in the spleens of BALB/c control mice versus BALB/c injected with anti-CCL2 after low dose infection. Mice were infected with $2x10^3$ CFU of bacteria, after appropriate time points animals were sacrificed, spleens removed and aliquots plated on BHI agar plates after homogenization.

3.5.2.2 Kinetics of changes in spleen cell composition of anti-CCL2 Abs treated mice after *L. monocytogenes* infection

To follow the changes in the composition of spleen cells in Abs injected animals, spleens were isolated from infected animals at appropriate time points and single spleen cell suspensions were stained for the surface markers characteristic for the main cell populations in this organ. Kinetics of cell populations from the Abs treated mice was compared with that from control animals.

Interestingly the main differences were visible only during the first two days PI, which is consistent with the hypothesis, that CCL2 function is dominating only during the early phase of infection (Figure 3.34). Also consistent with the data is the hypothesis that mainly macrophages are affected by CCL2 inhibition, since increase of ERTR-9^+ and MOMA-1^+ macrophage populations was delayed by 24 h in comparison to the untreated mice. $CD11c^+$, Gr-1^+ and Mac-1^+ cells display minor differences between control and Abs treated mice. Other populations including T cells ($CD3^+$), B cells ($B220^+$) and NK cells ($DX5^+$) were almost unaltered.

All data described above suggest that CCL2 has a particular function at the early stage of *Listeria* infection in BALB/c mice. Cells taking part in immediate response are mostly affected during the first two days. At later time points, lack of CCL2 might be compensated by other expressed chemokines.

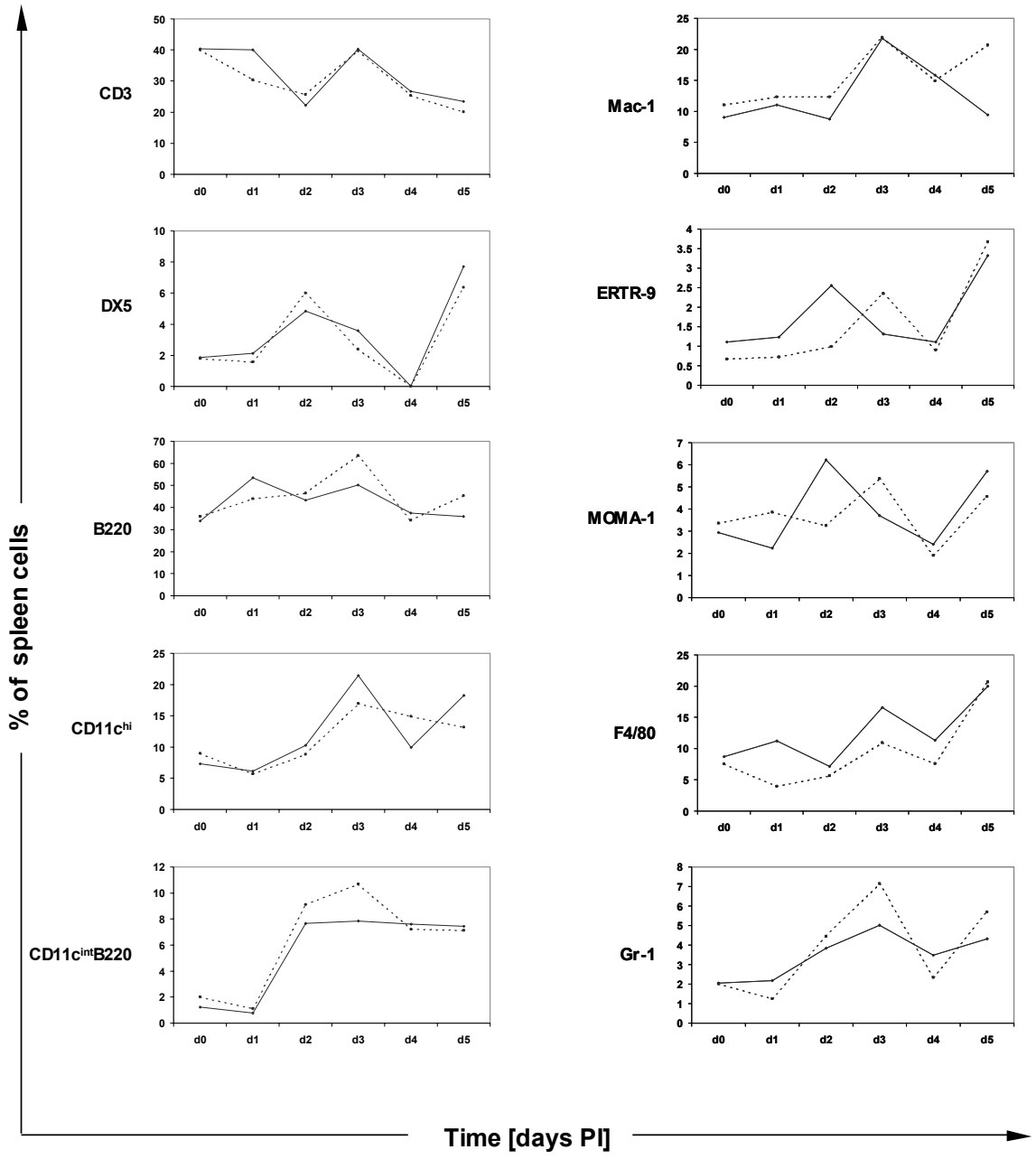


Figure 3.34 Changes in spleen cell populations after infection with low dose of *L. monocytogenes*. BALB/c mice treated with anti-CCL2 or PBS (control mice) were infected with a $2x10^3$ CFU of *Listeria*, at certain time points single cell suspensions were prepared from the spleens of infected animals. Cells were stained using antibodies recognizing surface markers characteristic for the different spleen cell populations. Biotinylated antibodies were revealed using SA-APC (Pharmingen, San Diego, CA). After washing and staining of the dead cells with propidium iodide (Sigma-Aldrich, Deisenhofen, Germany) alive cells were analyzed by FACSCalibur. Comparisons of the control mice (continuous line) and mice treated with Abs (dashed line) are showed.

4 Discussion

The major purpose of this work was to understand the complex immediate early events induced by *L. monocytogenes* during infection. Since the major target cell population of *Listeria* are macrophages and given that bacteria are known to trigger many signal transduction pathways in such infected macrophages, expression arrays were employed for large scale measurement of gene expression. Array analysis has been successfully applied to study interactions of other bacteria with host cells e.g. *Salmonella typhimurium* (Detweiler et al. 2001), *Pseudomonas aeruginosa* (Huang and Hazlett 2003) and *Chlamydia pneumoniae* (Virok et al. 2003). Additionally, two reports concerning infection of epithelial cells and the human promyelocytic cell line THP1 by *L. monocytogenes* are also known (Cohen et al. 2000;Baldwin et al. 2003). In these published experiments the number of induced genes was clearly higher (~500 and ~300 genes, respectively). In epithelial cells the genes regulated were mainly dependent on activation of NFκB. In the THP1 cell line mostly pro-inflammatory cytokines, chemokines and cell surface proteins were upregulated, similarly to the results obtained in this work. The differences in gene regulation that were observed between the cell types are not surprising and most likely depend on the host cell function.

In this work, gene expression pattern in *Listeria* infected murine J774 macrophages was tested. Regulation of genes was analyzed after 2 and 6 h using cDNA macroarray and microarrays. 35 genes were found to be significantly regulated on macroarrays and 100 genes on microarrays, respectively. Most of those genes encode for inflammatory cytokines and chemokines. Except for slight discrepancies, which could be due to the different sensitivity of these two arrays, the results obtained by the two approaches were consistent. In both methods more genes were specifically induced than repressed.

The lower number of induced genes in J774 in comparison to published results can be due to several reasons. The macroarray contains only a small number of genes in comparison to microarrays. In case of microarrays the statistics performed with the double determination might have deleted some of the data as not significant. On the other hand, *Listeria* might not activate J774 to the same extend as epithelial cells or THP1.

The majority of genes were regulated in a LLO dependent manner. In cells infected with a mutant of bacteria lacking LLO only few of the genes were upregulated, in comparison to wild type infection.

Surprisingly, IFNβ was also upregulated in a LLO dependent manner. *L. monocytogenes*, a Gram-positive bacterium, is known to induce signals mainly via TLR2 (Ireton and Cossart

1997;Cossart and Lecuit 1998), which does not induce IFNα/β (Braun and Cossart 2000). Hence, the signals induced by LLO obviously bypass or complement the deficiency of TLR2 to activate IFNα/β (Toshchakov et al. 2002).

The induction of CCL12 and CXCL10 also seemed to depend on LLO. CCL12 and CXCL10 have been shown to be activated via STAT1 pathway (Samsom et al. 1997;Kopydlowski et al. 1999;Toshchakov et al. 2002;Schilling et al. 2002). Since IFNα/β signal activates this pathway, it is not unreasonable to imagine that LLO through the induction of interferons may activate an autocrine loop to upregulate these chemokines. This does not, however, exclude the direct induction of chemokines by LLO.

Expression data obtained by arrays were confirmed for genes of interest by Real time-RT PCR and extended to other types of macrophages. Different kinetics of chemokine regulation was observed. The only cytokines that seemed to be induced in all these macrophage types were IFNβ and CXCL10. J774 macrophages turned out to be the most susceptible for infection and the most potent producers of chemokines. In contrast, BMDM seemed to be very weakly affected, only few chemokines were upregulated. This phenomenon could be due to the different phagocytic activities and different efficiency of killing of ingested bacteria. It is known that J774 have a low bactericidal activity, opposite to BMDM, which are especially bactericidal and kill most of the intracellular *Listeria* within the first four hours PI (de Chastellier and Berche 1994). Thus, BMDM might be equivalent to a subset with a different function than the subset represented by J774.

In vivo, most of the analyzed cytokines and chemokines were regulated as expected exclusively in the macrophage enriched adherent population. One could expect to observe the same signals also in the total spleen cell sample. This was however not the case most of the time, probably because the percentage of cells expressing the particular gene was too low to obtain a signal under these conditions.

Interestingly, expression of IFNα/β was restricted to a non-adherent cell population. Thus, macrophages do not produce this cytokines *in vivo* upon infection by Listeria in contrast to the *in vitro* experiments. The importance of IFNα/β during listerial infection is unclear since mice lacking the IFN receptor remain resistant against *Listeria* (van den Broek et al. 1995). On the other hand injection of IFNβ into infected mice has an adverse effect on the clearance of intracellular bacteria (Stockinger et al. 2002).

A similar discrepancy was observed for IL-6. *In vitro* IL-6 was strongly induced by *L. monocytogenes* infection in all the macrophages tested and was taken as indicator for the efficiency of infection. *In vivo* only a weak induction could be observed.

Furthermore, patterns of chemokine gene regulation in adherent cells were also different compared to *in vitro* data e.g. CCL5 was not upregulated *in vivo*, while it was strongly induced *in vitro*. This was probably due to a constitutively high expression in spleen that is not increased upon infection or cells able to produce this chemokine were not stimulated upon infection.

All these differences demonstrate the complexity that is encountered during such experiments. *In vitro* cells of one type are studied while *in vivo* cell-cell interactions will influence the reactions. Some cells might also produce certain cytokines constitutively for homeostasis. On the other hand, cells might be infected that are unable to express certain cytokines. Besides, only a small percentage of cells is infected which might explain low signals of particular molecules.

Since the expression of chemokines was restricted to the enriched in macrophages adherent cell population, quantitation of chemokine gene expression by Real-Time RT-PCR was carried out only with this population. That confirmed the results obtained with RT-PCR and allowed an estimation of the level of regulation. Interestingly, the early expressed chemokines CCL2, CCL7 and CCL12 are also known as members of 'Monocyte Chemoattracting Protein' subfamily. All of these chemokines signal through the same receptor CCR2 and are known for their macrophage chemotactic capacity. (Morrison et al. 2003) (Lee et al. 2003).

The strongest 1400 fold transient upregulation in adherent cell population showed CCL2, considered to be the most potent macrophage chemoattractant and activator (Rollins 1997;Lee et al. 2003). This is also astonishing, since at this time point relatively few cells are infected. The expression however seems to be brief, reaching a peak at 4 h PI, and decreasing almost to background level by 24 hours. This could be the consequence of transient gene activation in infected cells or the killing of such cells by *Listeria*. The induction of CCL2 together with CCL7, another macrophage specific chemoattractant, is in agreement with the finding that macrophages are the earliest mobilized cells under theses conditions.

Most of the remaining chemokines tested, showed low or no induction during the early phase of infection, but could readily be detected at 24 hrs PI. A few of them, like CXCL2 and CXCL10 display fold increase factors between 180 and 1600. The strong upregulation of

CXCL10 is particularly interesting, since this chemokine is known as an attractant of IFNγ producing T cells (Schilling et al. 2002) and activated memory T cells (Cohen et al. 2000). This explains the presence of T cells within the macrophage clusters at the late time points.

In order to follow the course of infection in the spleen of infected BALB/c mice confocal analysis of antibody stained cryosections was employed. The most impressive change in the spleen was the infiltration of Mac-1^+ cells. Mac-1^+ cells, which consist mainly of macrophages, but also of granulocytes and a subpopulation of DCs, start to migrate by 4 h PI. At 24 h PI these cells form huge clusters around infection foci. Such a massive macrophage migration is most likely the consequence of the strong upregulation of macrophage specific chemoattractants, especially CCL2. The only cells found to be infected as early as 4 h PI are ERTR-9^+ macrophages. Infection is probably restricted to this population even at later time points of infection such as 24 h PI. At this time ERTR-9^+ macrophages are found in the macrophage clusters together with Mac-1^+ cells, some pDCs and $CD3^+$ cells. Hence ERTR-9^+ cells are most likely responsible for release of chemokines attracting other macrophages, DCs and T cells to the place of infection.

The second subpopulation of marginal zone macrophages bearing the MOMA-1 marker seemed not to be involved in bacteria uptake and subsequent macrophage clustering. No bacteria were found to be associated with them and after 24 h these macrophages had migrated into the B cell follicle.

It is astonishing that two populations of macrophages ERTR-9^+ and MOMA-1^+ that anatomically colocalize, could display such different functions. It was not known before that ERTR-9^+ cells are the only macrophages in MZ that take up *Listeria*. Probably, these cells, in contrast to MOMA-1^+ macrophages, express receptors facilitating phagocytosis e.g. CR3 receptor (Mac-1) responsible for opsonin dependent phagocytosis is expressed one some ERTR-9^+ cells (data not shown). Since macrophages from the MZ were found to have CR3 receptor (Brown 1991;Aichele et al. 2003) and staining of cryosections never revealed Mac-1^+ cells inside B cell follicle, ERTR-9^+ cells are the only MZ macrophages expressing this receptor. In addition, ERTR-9 is a C-lectin (Geijtenbeek et al. 2002;Kang et al. 2003) that might be able to bind to the cell wall of *Listeria*. This would explain the exclusive infection of ERTR-9^+ cells by *Listeria*.

It remains unclear what drives MOMA-1^+ cells into the follicles and what function they fulfill there. Therefore, it would be suitable to sort these macrophages from the infected mice to globally analyze genes expressed upon infection. This is now possible since in the meantime

protocols for the amplification of RNA have been established allowing array analysis even for small cell populations. This analysis could uncover receptors possibly involved in MOMA-1^+ migration or other receptors responsible e.g. for antigen presentation. Similar analysis should be performed using isolated ERTR-9^+ cells.

Cell clustering might be a reaction helpful in the defense of the host against the bacteria. On the other hand, clustering might also enable *Listeria* to infect more phagocytic cells and effectively multiply in their cytoplasm. Hence, early CCL2 production in macrophages might be triggered by bacteria in order to facilitate further spreading. Since such an effect requires direct contact between cell and pathogen, CCL2 production should be upregulated only in infected cells, in this case ERTR-9^+ cells. In order to test this possibility the main populations of macrophages and DCs from the spleen were sorted and analyzed for chemokine gene expression. As expected the main producers of CCL2 at 4 h PI were ERTR-9^+ macrophages. However, at later time points also MOMA-1^+ and F4/80^+ macrophages as well as DCs and the subpopulation of pDCs also appeared to produce this chemokine. Nevertheless, the finding that only ERTR-9^+ cells are able to produce CCL2 at 4 h PI correlates nicely with the fact that only these cells contain bacteria at this early stage of infection.

The analysis of sorted cells revealed a discrepancy with the data obtained before with adherent spleen cells. While in adherent cell population CCL2 production appeared extremely high at 4 h PI and ceased at later time points to almost background values, in sorted populations it remained upregulated and additional cells started to produce it.

This discrepancy is most likely due to the procedure that was used to prepare the cells. Adherent spleen cell are a heterogeneous population. Their composition might change depending on the activation state of particular cells and influx of more phagocytic cells into the spleen. Thus, the CCL2 producing cells might be out numbered by non producers at 24h. In addition, at this time point infected ERTR-9^+ macrophages might be killed either by *Listeria* directly or by cytotoxic NK or T cells, since their number is extremely reduced at this time point. Therefore, the signal in the total adherent population might decrease in spite of its upregulation at the single cell level.

ERTR-9^+ macrophages were also the early producers of other chemokines like CXCL2 responsible for neutrophil attraction and degranulation and CXCL10 attractor and activator of Th1 cells. Interestingly, plasmacytoid DCs were the second cell population activated early during listerial infection. These cells are found in the clusters and contribute to the CCL2 and CXCL10 production 4 h PI, although the increase of expression was very low. As expected,

MOMA-1^+ macrophages seem to have another role during *Listeria* infection than production of chemokines. F4/80^+ macrophages of the red pulp appear to play no role during the early stage of infection. Both cell types started to upregulate expression of chemokines only after 24 h. At this time point a high bacterial load might have led to some deregulation.

IFNβ is not produced by any of these isolated cell populations confirming the data obtained before. Similarly, all populations of sorted cells constitutively expressed CCL3, CCL4 and CCL5. This is consistent with regulation of these genes in adherent spleen cell population from infected mice.

A high dose of *Listeria* was used to facilitate analysis of immediate host reactions. Since this dose might cause peculiar host reactions, the low dose of bacteria was used to compare the chemokine pattern in these two situations. Although delayed the pattern of chemokine expression was consistent with the pattern obtained using a high dose. This correlates well with a delay in the splenic architecture rearrangement.

Detection of CCL2 during immediate responses against the low dose was below detection using standard RT-PCR reaction. In order to confirm the appearance of CCL2 during the early stage of infection, semi-nested PCR had to be performed. Increased sensitivity revealed CCL2 induction timing similar to that of a high dose infection. However, as seen above, this will also have to be studied using sorted cells to reveal the exact pattern of expression.

It is yet not understood why during outbreaks of listeriosis some individuals can come up with fatal disease while others that were also exposed to the pathogen do not suffer from even minor symptoms. Thus, resistance and susceptibility is a major issue in understanding the pathogenicity of microorganisms. Murine listeriosis represents a well established model for this phenomenon. Certain mouse strains are distinguished by their grade of susceptibility towards an intravenous infection. This state should be reflected in their early defense response, in particular their chemokine and cytokine response, since these molecules are part of the regulatory cascade directing host reactions. Indeed, the three mouse strains investigated displayed significant differences in their expression pattern of chemokines and cytokines.

In C57Bl/6 mice CCL2 was very weakly upregulated in comparison to BALB/c and not found before 24 h PI. Since early expression of CCL2 is associated with Th2 responses (Chensue et al. 1996;Karpus et al. 1997), the expression pattern of this gene in C57Bl/6 mice is consistent with their Th1 phenotype. Other chemokines like CCL3, CCL4, CXCL2 and CXCL10 were

upregulated earlier compared to BALB/c. Interestingly; IFNγ was also found upregulated early during this response.

DBA/2 mice, known to be very susceptible to *Listeria* infection, were the only strain where IFNβ was upregulated in the adherent cell population. The significance of this observation is unclear. IFNβ can have attenuating effects on inflammation (Teige et al. 2003) but might be also involved in the reaction against bacterial products (Karaghiosoff et al. 2003). CCL2 dominating early in BALB/c mice was upregulated late in DBA/2 mice and at comparably low level. Upregulation IFNγ and the expression pattern of CCL12 and CXCL10 also indicate that the direction of the immune response in these mice is biased towards the Th1 pathway.

The given data show that susceptibility against *Listeria* is a highly complex phenomenon. No clear cut difference in the expression of a cytokine or chemokine was encountered that could be declared as responsible for the resistant or susceptible phenotype. One also has to bear in mind that the analysis was restricted to mixed cell populations. The experiments discussed above demonstrated that such experiments needed to be extended to sorted cells, since the dynamics of the changes in composition are not taken into account otherwise. Nevertheless, the results obtained already suggest the direction for future investigations.

In BALB/c mice, CCL2 was found to be the dominant chemokine at the early stage of *Listeria* infection. Thus, one could imply that this chemokine is partly responsible for the susceptibility of this mouse strain. To test this hypothesis, CCL2 activity was blocked in mice using antibodies before application of *L. monocytogenes*. Mice depleted in CCL2 and infected with a high dose of *Listeri*a showed a reproducible two fold reduction of bacteria in the spleen. This would be consistent with the assumption that CCL2 interferes with the inflammatory response against *Listeria*.

The expression patterns of chemokines and cytokines in the adherent spleen populations from CCL2 depleted mice were also consistent with the above hypothesis. All chemokines and cytokines tested including CCL2 were expressed at apparently higher levels and a few of them like IFNγ, CCL7, CCL12 and CXCL10 could be detected earlier compared to untreated BALB/c mice. Stronger CCL7 and CCL12 expression might be a compensating mechanism for the lack of CCL2 activity to attract macrophages to the infectious foci. These chemokines signal via the same receptor as CCL2 (CCR2) but also via CCR1 and CCR3, and therefore are more prone to drive strong inflammatory reactions. The early upregulation of IFNγ and CXCL10 is also consistent with this idea. Unexpectedly, adherent spleen cell population from

these mice upregulated IFNβ. It is possible that mechanisms compensating the lack of CCL2 trigger different signaling pathways in host cells or attract and activate other subsets of cells e.g. plasmacytoid DCs.

It is postulated that CCL2 is indispensable for macrophage recruitment only during the early phase of an infection and that compensatory factors like other chemokines cannot overcome this defect. At later time points the function of CCL2 might be replaced by other chemokines (Warmington et al. 1999). To test this possibility mice depleted in CCL2 were infected with a low dose of *Listeria*. Spleen cell composition in these mice differed from untreated controls only for the first two days PI. Cells like granulocytes and Mac-1^+ cells displayed minor differences, while T cells, NK cells, DCs and B cells were almost unaltered. Most affected by the block of CCL2 were ERTR-9^+ and MOMA-1^+ macrophages. Their increase in numbers was delayed by 24 h in CCL2 depleted mice in comparison to untreated BALB/c mice. This transient delay could be due to the exhaustion of the antibody, but more likely the expression of other chemoattractants for macrophages compensate the lack of CCL2. These possibilities could not be distinguished within the time frame of this work.

Why does the lack of CCL2 consistently reduce the numbers of bacteria in the spleen under high or low dose infection conditions? Since macrophages are the main host cells for *Listeria*, fewer macrophages at infectious foci could minimize the bacterial dissemination. In addition, the altered chemokine milieu in CCL2 depleted mice might result in more aggressive macrophages migrating towards these foci, since distinct listericidal potency has been observed before in differently activated macrophages (Fleming and Campbell 1997;Conte et al. 2002).

Taken together, a model for early events during *Listeria* infection could be established, which is schematically depicted in Figure 4.1. Infected ERTR-9^+ macrophages producing CCL2 would be the central players that induce most subsequent steps by chemoattraction of other subsets of cells. In addition, secreted bacterial virulence factors also might play a significant role in these reactions. This model should explain the sequential and rapid restructuring observed in the spleen in the early stages of *L. monocytogenes* infection.

Although not without limitations; obtained data show that *in vitro* global analysis of gene expression is a reliable approach of identifying significantly regulated genes for further *in vivo* studies. This approach could be suitable especially in studying the complex host-pathogen interactions during bacterial infections in systems where *in vivo* studies are limited, like in the human. In such cases, global analysis of regulated genes upon *in vitro* infection

should reveal molecules and cells that could subsequently be investigated in clinical specimens. Complementing studies *in vitro* should then allow the unraveling of cellular and molecular interactions that take place. Therefore, this study should also be taken as an example where this approach has successfully been applied.

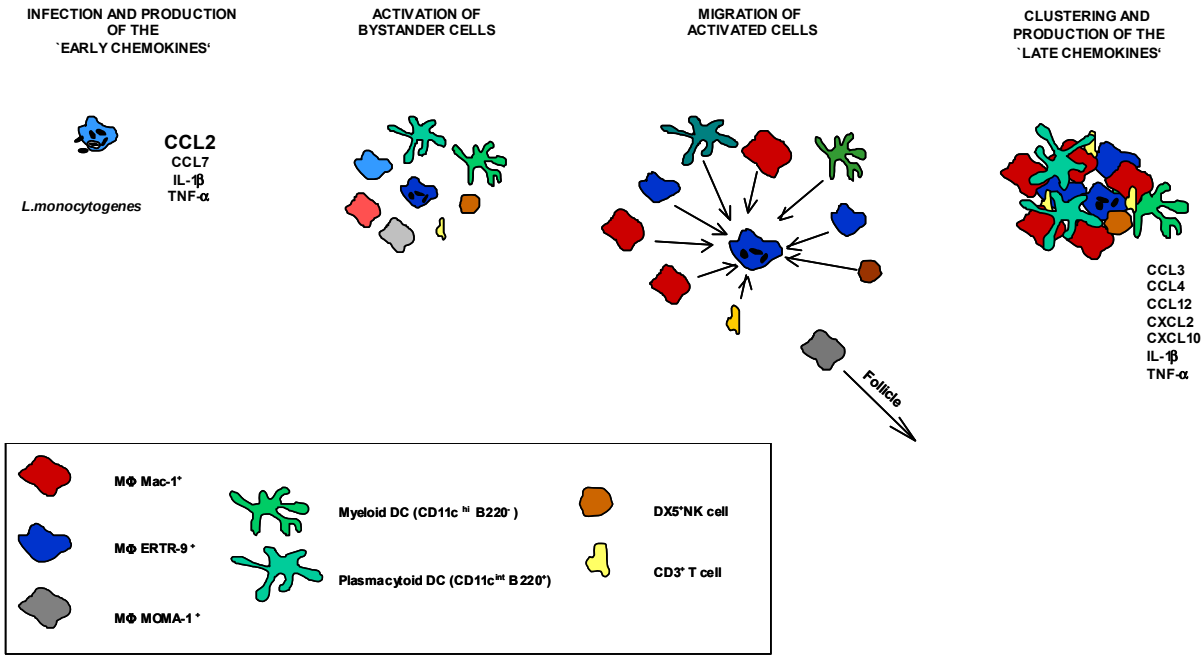


Figure 4.1 Sequention of events during early *L. monocytogenes* infection. Infection and subsequent activation of ERTR-9+ macrophages results in a transient expression of CCL2 and induction of other cytokines. Neighboring macrophages and dendritic cells, including plasmacytoid dendritic cells, are attracted and activated. This results in the attenuation of CCL2 production, while giving into the expression of 'late' chemokines. These chemokines amplify responses through further recruitment and activation of macrophages and dendritic cells as well as NK cells and T cells, which then formed the large clusters in the spleen . Cytokines produced by these clusters drive probably anti-*Listeria* reactions into a Th1 pathway.

5 Summary

Immediate events during infection of mice with *L. monocytogenes* which might be decisive for the course of infection and host reactions are still ill defined. Most likely this is due to the low numbers of cells infected during this time and the difficulty to isolate such cells. Therefore, a novel strategy was employed here. First, macrophages were infected *in vitro* and genes regulated were analyzed by gene expression arrays and Real Time RT-PCR. Mainly inflammatory cytokines and chemokines were induced in these cells upon infection. The gene expression profiles were heterogeneous amongst the different macrophages employed, most likely reflecting a heterogeneity that might be also encountered *in vivo*.

To confirm cytokine and chemokine regulation *in vivo*, BALB/c mice were infected with *L. monocytogenes* using a high dose of bacteria. Induction of cytokines and chemokines was mainly restricted to the adherent macrophage enriched spleen cell population and showed interesting discrepancies with the in vitro data. Quantitation of the expression level by Real-Time RT-PCR revealed that already at 4 h PI genes coding for macrophage chemoattractants were upregulated with the most significant expression of CCL2. Most other inflammatory chemokines required 24 h most for upregulation. These results were corroborated using sorted macrophages and dendritic cells from infected mice. Early expression of CCL2 could be confirmed. It was restricted mainly to the macrophage population displaying the marker ERTR-9.

This was consistent with immunohistological analysis. Bacteria were to the largest extend associated only with ERTR-9^+ marginal zone macrophages, even in the late phase of infection. Furthermore, ERTR-9^+ cells might be responsible for the activation of other types of cells, either via the secreted cytokines and chemokines, or through direct cell-cell contact. Indeed, large extensions of ERTR-9^+ macrophages interact with other types of macrophages. Thus, infected ERTR-9^+ macrophages might be the condensation foci for the reorganization observed in the spleen.

In contrast, MOMA-1^+ MZ macrophages seemed to play no role in direct anti-*Listeria* response. These cells were not infected and were found after 24 h in B cell follicles. The function of these cells remains unclear.

Infection with a low sublethal dose of bacteria confirmed the pattern of regulated chemokines and the migration pattern of cells in the spleen observed with a high dose, only the time required for completion was substantially longer. Again CCL2 expression was immediately upregulated after infection.

In order to study the chemokine expression pattern in mice with different susceptibility to *Listeria* infection, resistant C57Bl/6 and highly susceptible DBA/2 mice were infected. Indeed, cytokine and chemokine regulation patterns were altered in spleen cells of these mice in comparison to BALB/c. Spleen architecture was also different, since macrophage clusters were smaller in C57Bl/6 and DBA/2 mice. This demonstrates that the different degrees of susceptibility depend upon complex interactions.

Since CCL2 has been shown to dominate the early phase of *Listeria*-induced reactions in BALB/c mice, the importance of this chemokine was investigated *in vivo* using a neutralizing antibody. The employed experiments revealed an essential role of CCL2 in the early increase of marginal zone macrophages in the spleen. Chemokine and cytokine pattern in infected BALB/c mice depleted of CCL2 also exhibited changes. The immune reactions seemed to be more directed towards the Th1 pathway. Number of *Listeria* in antibody treated animals were consistently lower then in controls. Thus induction of CCL2 and the attraction of macrophages might facilitate multiplication of *L. monocytogenes* and act as an immune escape mechanism of these pathogens.

6 References

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ABBREVIATIONS

Abs	antibodies
Ag	antigen
APC	antigen presenting cells
BHI	brain heart infusion
bp	base pair
BSA	bovine serum albumin
CAM	cell adhesion molecule
CCP	classical complement cascade
cDNA	complementary deoxyribonucleic acid
CD	cluster of differentiation
CFU	colony forming units
CNS	central nervous system
CpG	cytosine-phosphate-guanosine
CR	complement receptor
DCs	dendritic cells
DNA	deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
Fc	crystallizing fragment
FACS	fluorescence activated cell sorting
FcR	Fc receptor
FCS	fetal calf serum
g	gravital acceleration
GM-CSF	granulocyte macrophage-colony stimulatory factor
h	hour
hi	high
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's modified Eagle's medium
Inl	internalin
iNOS	inducible nitric oxide synthase
int	intermediate

iv	intravenous
LD	lethal dose
LD50	dose lethal for the 50% of infected mice
LLO	listeriolysin O
LPS	lipopolisaccharide
LT	leucotriene
LTA	lipoteichoic acid
MALT	mucosa-associated lymphoid tissue
MARCO	macrophage receptor with a collagenous structure
MBP	mannose-binding protein
MCP	macrophage chemotactic protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory chemokine
MM	marginal metallophilic macrophages
MOI	multiplicity of infection
MZ	marginal zone
MZM	marginal zone macrophages
NK	natural killer
OD	optical density
PALS	periarteriolar lymphoid sheath
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PC	phosphorylcholine
PCR	polymerase chain reaction
pDCs	plasmacytoid DCs
PG	prostaglandin
PI	post infection
PKC	protein kinase C
PLC	phospholipase C
PRRs	pattern recognition receptors
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates

RT	room temperature
RT-PCR	reverse transcription dependent polymerase chain reaction
SA	streptavidin
SR	scavenger receptor
SRCR	Scavenger Receptors Cysteine Rich
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor

Addendum

Fold Change				
2h	**6h**	**Gene name**	**Classification**	**Genebank**
	2.1	glycoprotein 49 A	membrane fraction	M65027
	-3.8	lymphoblastomic leukemia	nucleus DNA binding cell growth and maintenance	X57687
	-3.5	lymphoblastomic leukemia	nucleus DNA binding cell growth and maintenance	X57687
	12.5	matrix metalloproteinase 13	collagenase extracellular matrix metalloendopeptidase	X66473
	3	ESTs Weakly similar to lysophospholipase I [M.musculus]		AA816121
6.6	12	small inducible cytokine subfamily member 2	chemotaxis signal transduction inflammatory response	X53798
	2.5	DNA segment Chr 1 Lubeck 1		M55219
	3.4	DNA segment Chr 1 Lubeck 1		M55219
	49	interleukin 6		X54542
	16.9	B-cell leukemia/lymphoma 3		M90397
	2.1	Fc receptor IgG low affinity IIb	plasma membrane lymphocyte antigen	M31312
	3.1	Jun-B oncogene	nucleus DNA binding regulator of transcription	U20735
2.2	3.2	small inducible cytokine A3	chemotaxis signal transduction inflammatory response	J04491
	4.2			D84196
	-4.1	calmodulin binding protein 1		AF062378
	16	serum amyloid A 3	acute-phase response	X03505
	3.3	small inducible cytokine A2	chemotaxis signal transduction inflammatory response	M19681
	25.2	growth arrest and DNA-damage-inducible 45 beta	apoptosis histogenesis and organogenesis	X54149
	46.8	serine protease inhibitor 2-1	serpin serine protease inhibitor	M64085
	5.7	T-cell specific GTPase		L38444
	3.8			U23778
	2.1	ATP-binding cassette sub-family B MDR/TAP member 2	ABC transporter	U60020
	4.5	guanylate nucleotide binding protein 3	GTPase cytosol	AW047476
	3.4			AW122677
	3.2	RIKEN cDNA 9130009C22 gene		AA959954
9.5	63	interleukin 1 beta	immune response cell cycle regulator inflammatory response	M15131
	2.8	RIKEN cDNA 5031415C07 gene		AA822898
	2.3	RIKEN cDNA 4930534K13 gene		AW125713
2.1	2.9	activating transcription factor 3	nucleus DNA binding transcription factor	U19118
2.7	4.5	activating transcription factor 3	nucleus DNA binding transcription factor	U19118
	9.5	ESTs		AA204579
	-5.4	glutathione peroxidase 3	peroxidase reaction glutathione peroxidase	AI120844
	2.3	ESTs		AI852578
	3.3	proviral integration site 1	ATP binding protein, serine/threonine kinase	AA764261
	15.5	guanylate nucleotide binding protein 2	GTPase	AJ007970
3.1	4.2	prostaglandin-endoperoxide synthase 2	prostaglandin-endoperoxide synthase	M88242
	8.3	interferon gamma inducible protein 47 kDa		M63630
	2.2	beta-1 3-N-acetylglucosaminyltransferase 1		AW260308

	-5.5	butyrate response factor 2	nucleus DNA binding	AA960603
	2.4	MAP kinase-activated protein kinase 2	ATP binding protein, serine/threonine kinase	X76850
	2.5	tumor necrosis factor alpha-induced protein 2		L24118
	2.7	lipocalin 2	transport	X81627
	5	T-cell death associated gene		U44088
	3.3	CCAAT/enhancer binding protein C/EBP delta	nucleus DNA binding transcription regulation	X61800
	2.1	interferon gamma induced GTPase	GTPase cytosol	U53219
	10.6	interleukin 15 receptor alpha chain	cell surface receptor	U22339
	6.9	DNA segment Chr 14 University of California at Los Angeles 3		AV003873
	2.5	Mus musculus adult male hippocampus cDNA RIKEN full-length enriched library clone 2900034J12 full insert sequence		AV152244
	4.1	growth arrest and DNA-damage-inducible 45 beta	apoptosis histogenesis and organogenesis	AV138783
2.9	6.2	cytokine inducible SH2-containing protein 3		AV374868
	2.6	glycoprotein 49 B	membrane fraction	U05265
	15.6	cytokine inducible SH2-containing protein 3		U88328
	2.8	inhibitor of kappa light polypeptide gene enhancer in B-cells kinase epsilon	IkB kinase	AB016589
	3	serum-inducible kinase	ATP binding protein serine/threonine kinase	M96163
	4.3	zinc finger protein 36	nucleus DNA binding nucleic acid binding	X14678
	8.5	colony stimulating factor 2 granulocyte-macrophage		X03020
	5.6	tumor necrosis factor receptor superfamily member 5	membrane fraction	M83312
	6.9	histidine decarboxylase cluster		X57437
	2.5	hemopoietic cell kinase	ATP binding protein myristylation protein tyrosine kinase	J03023
	2.3	complement component 3	complement activation\ classical \ alternative pathway	K02782
	4.4	RIKEN cDNA 1810047I05 gene		AF030185
	10.8	small inducible cytokine A12	chemotaxis signal transduction inflammatory response	U50712
	2.8	RIKEN cDNA 5031415C07 gene		AA261092
5.1	6.5	small inducible cytokine B subfamily CXC member 10	chemotaxis immune response inflammatory response	M33266
-2.5		RIKEN cDNA 1300019I03 gene		AI845584
	30.4	interleukin 1 receptor antagonist	cell surface receptor	L32838
	5.7	tumor necrosis factor ligand superfamily member 10	apoptosis membrane fraction	U37522
	2.2	scavenger receptor	endocytosis membrane fraction	M59446
20.6	79.4	interferon beta fibroblast	defense response	K00020
4.1	6.7	small inducible cytokine A4	chemotaxis signal transduction inflammatory response	X62502
	7.2	Tnf receptor-associated factor 1		L35302
	-2.1	cyclin D1	cyclin - cell cycle control	AI849928
	2.7	immediate early response 3	membrane fraction	X67644
	3.2	RIKEN cDNA 2810468L03 gene		AW124934
	6.5	interleukin 1 alpha	immune response cell cycle regulator inflammatory response	M14639
7.6	20.7	small inducible cytokine A7	chemotaxis signal transduction inflammatory response	X70058
	2.2	cyclin-dependent kinase inhibitor 1A P21	nucleus cell cycle cyclin-dependent protein kinase inhibitor	AW048937
	2.7	tumor necrosis factor receptor superfamily member 1b	necrosis membrane fraction cell proliferation	X87128
	4.4	clusterin		D14077

	-3.4	ESTs Highly similar to p53 regulated PA26-T2 nuclear protein [H.sapiens]		AI843106
	15.3	guanylate nucleotide binding protein 1	GTP binding immune response	M55544
	-2.3			X60980
	2.2	SAC2 supressor of actin mutations 2 homolog like S. cerevisiae	TAP binding protein	AF110520
	15.3	interferon-inducible GTPase	GTPase	AJ007971
3.5	7.5	ESTs		C78513
	5.3	RIKEN cDNA 3830417M17 gene		AI642662
	2.6	cyclin-dependent kinase inhibitor 1A P21	nucleus cell cycle cyclin-dependent protein kinase inhibitor	U09507
	3.1	CD14 antigen	plasma membrane post-translational membrane targeting	X13333
	20.1	small inducible cytokine A5	chemotaxis signal transduction inflammatory response	AF065947
3.1	9.2	immunoresponsive gene 1		AI323667
5.2	40.7	immunoresponsive gene 1		L38281
4.3	5.9	Mus musculus mRNA for MAIL complete cds		AA614971
	3.3	proviral integration site 1	ATP binding protein,serine/threonine kinase	M13945
	4.6	tumor necrosis factor alpha-induced protein 3	apoptosis cytoplasm DNA binding	U19463
	-11.2	TGFB inducible early growth response	DNA binding	AF064088
	-14.3	TGFB inducible early growth response	DNA binding	AF064088
14.4		CD83 antigen	plasma membrane	AF001036